

ITC X-Vaccine and virus studies

Amarasinghe G. K., De Guzman R. N., Turner R. B., Chancellor K. J., Wu Z. R., and Summers M. F. (2000) NMR structure of the HIV-1 nucleocapsid protein bound to stem-loop SL2 of the psi-RNA packaging signal. Implications for genome recognition. *J Mol Biol* **301**, 491-511.

Abstract: The RNA genome of the human immunodeficiency virus type-1 (HIV-1) contains a approximately 120 nucleotide Psi-packaging signal that is recognized by the nucleocapsid (NC) domain of the Gag polyprotein during virus assembly. The Psi-site contains four stem-loops (SL1-SL4) that possess overlapping and possibly redundant functions. The present studies demonstrate that the 19 residue SL2 stem-loop binds NC with affinity ($K_d=110(+/-50)$ nM) similar to that observed for NC binding to SL3 ($K(d)=170(+/-65)$ nM) and tighter than expected on the basis of earlier work, suggesting that NC-SL2 interactions probably play a direct role in the specific recognition and packaging of the full-length, unspliced genome. The structure of the NC-SL2 complex was determined by heteronuclear NMR methods using $(15)N,(13)C$ -isotopically labeled NC protein and SL2 RNA. The N and C-terminal "zinc knuckles" (Cys-X(2)-Cys-X(4)-His-X(4)-Cys; X=variable amino acid) of HIV-1 NC bind to exposed guanosine bases G9 and G11, respectively, of the G8-G9-U10-G11 tetraloop, and residues Lys3-Lys11 of the N-terminal tail forms a 3(10) helix that packs against the proximal zinc knuckle and interacts with the RNA stem. These structural features are similar to those observed previously in the NMR structure of NC bound to SL3. Other features of the complex are substantially different. In particular, the N-terminal zinc knuckle interacts with an A-U-A base triple platform in the minor groove of the SL2 RNA stem, but binds to the major groove of SL3. In addition, the relative orientations of the N and C-terminal zinc knuckles differ in the NC-SL2 and NC-SL3 complexes, and the side-chain of Phe6 makes minor groove hydrophobic contacts with G11 in the NC-SL2 complex but does not interact with RNA in the NC-SL3 complex. Finally, the N-terminal helix of NC interacts with the phosphodiester backbone of the SL2 RNA stem mainly via electrostatic interactions, but does not bind in the major groove or make specific H-bonding contacts as observed in the NC-SL3 structure. These findings demonstrate that NC binds in an adaptive manner to SL2 and SL3 via different subsets of inter and intra-molecular interactions, and support a genome recognition/packaging mechanism that involves interactions of two or more NC domains of assembling HIV-1 Gag molecules with multiple Psi-site stem-loop packaging elements during the early stages of retrovirus assembly.

Amarasinghe G. K., Zhou J., Miskimon M., Chancellor K. J., McDonald J. A., Matthews A. G., Miller R. R., Rouse M. D., and Summers M. F. (2001) Stem-loop SL4 of the HIV-1 psi RNA packaging signal exhibits weak affinity for the nucleocapsid protein. structural studies and implications for genome recognition. *J Mol Biol* **314**, 961-970.

Abstract: Encapsidation of the genome of the human immunodeficiency virus type-1 (HIV-1) during retrovirus assembly is mediated by interactions between the nucleocapsid (NC) domains of assembling Gag polyproteins and a approximately 110 nucleotide segment of the genome known as the Psi-site. The HIV-1 Psi-site contains four stem-loops (SL1 through SL4), all of which are important for genome packaging. Recent isothermal titration calorimetry (ITC) studies have demonstrated that SL2 and SL3 are capable of binding NC with high affinity (K_d approximately 140 nM), consistent with proposals for protein-interactive functions during packaging. To determine if SL4 may have a similar function, NC-interactive studies were conducted by NMR and gel-shift methods. In contrast to previous reports, we find that SL4 binds weakly to NC ($K_d=(+/-14)$ μ M), suggesting an alternative function. NMR studies indicate that the GAGA tetraloop of SL4 adopts a classical GNRA-type fold (R=purine, N=G, C, A or U), a motif that stabilizes RNA tertiary structures in other systems. In combination with previously reported gel mobility studies of Psi-site deletion mutants, these findings suggest that SL4 functions in genome recognition not by binding to Gag, but by stabilizing the structure of the Psi-site. Differences in the affinities of NC for SL2, SL3 and SL4 stem-loops can now be rationalized in terms of the different structural properties of stem loops that contain GGNG (SL2 and SL3) and GNRA (SL4) sequences.

Appleton B.A., Brooks J., Loregian A., Filman D.J., Coen D.M., and Hogle J.M. (2006) Crystal structure of the cytomegalovirus DNA polymerase subunit UL44 in complex with the C terminus from the catalytic subunit. Differences in structure and function relative to unliganded UL44. *J Biol Chem.* **281**, 5224-32.

Abstract: The human cytomegalovirus DNA polymerase is composed of a catalytic subunit, UL54, and an accessory protein, UL44, which has a structural fold similar to that of other processivity factors, including herpes simplex virus UL42 and homotrimeric sliding clamps such as proliferating cell nuclear antigen. Several specific residues in the C-terminal region of UL54 and in the "connector loop" of UL44 are required for the association of these proteins. Here, we describe the crystal structure of residues 1-290 of UL44 in complex with a peptide from the extreme C terminus of UL54, which explains this interaction at a molecular level. The UL54 peptide binds to structural elements similar to those used by UL42 and the sliding clamps to associate with their respective binding partners. However, the details of the interaction differ from those of other processivity factor-peptide complexes. Crucial residues include a three-residue hydrophobic "plug" from the UL54 peptide and Ile(135) of UL44, which forms a critical intramolecular hydrophobic anchor for interactions between the connector loop and the peptide. As was the case for the unliganded UL44 structure, the UL44-peptide complex forms a head-to-head dimer that could potentially form a C-shaped clamp on DNA. However, the peptide-bound structure displays subtle differences in the relative orientation of the two subdomains of the protein, resulting in a more open clamp, which we predicted would affect its association with DNA. Indeed, filter binding assays revealed that peptide-bound UL44 binds DNA with higher affinity. Thus, interaction with the catalytic subunit appears to affect both the structure and function of UL44.

Bacha U., Barrila J., Velazquez-Campoy A., Leavitt S. A., and Freire E. (2004) Identification of novel inhibitors of the SARS coronavirus main protease 3CLpro. *Biochemistry* **43**, 4906-4912.

Abstract: SARS (severe acute respiratory syndrome) is caused by a newly discovered coronavirus. A key enzyme for the maturation of this virus and, therefore, a target for drug development is the main protease 3CL(pro) (also termed SARS-CoV 3CL(pro)). We have cloned and expressed in *Escherichia coli* the full-length SARS-CoV 3CL(pro) as well as a truncated form containing only the catalytic domains. The recombinant proteins have been characterized enzymatically using a fluorescently labeled substrate; their structural stability in solution has been determined by differential scanning calorimetry, and novel inhibitors have been discovered. Expression of the catalytic region alone yields a protein with a reduced catalytic efficiency consistent with the proposed regulatory role of the alpha-helical domain. Differential scanning calorimetry indicates that the alpha-helical domain does not contribute to the structural stability of the catalytic domains. Analysis of the active site cavity reveals the presence of subsites that can be targeted with specific chemical functionalities. In particular, a cluster of serine residues (Ser139, Ser144, and Ser147) was identified near the active site cavity and was susceptible to being targeted by compounds containing boronic acid. This cluster is highly conserved in similar proteases from other coronaviruses, defining an attractive target for drug development. It was found that bifunctional aryl boronic acid compounds were particularly effective at inhibiting the protease, with inhibition constants as strong as 40 nM. Isothermal titration microcalorimetric experiments indicate that these inhibitors bind reversibly to 3CL(pro) in an enthalpically favorable fashion, implying that they establish strong interactions with the protease molecule, thus defining attractive molecular scaffolds for further optimization.

Barbieri C. M., Li T. K., Guo S., Wang G., Shallop A. J., Pan W., Yang G., Gaffney B. L., Jones R. A., and Pilch D. S. (2003) Aminoglycoside complexation with a DNA.RNA hybrid duplex: the thermodynamics of recognition and inhibition of RNA processing enzymes. *J Am Chem Soc* **125**, 6469-6477.

Abstract: Spectroscopic and calorimetric techniques were employed to characterize and contrast the binding of the aminoglycoside paromomycin to three octamer nucleic acid duplexes of identical sequence but different strand composition (a DNA.RNA hybrid duplex and the corresponding DNA.DNA and RNA.RNA duplexes). In addition, the impact of paromomycin binding on both RNase H- and RNase A-mediated cleavage of the RNA strand in the DNA.RNA duplex was also determined. Our results reveal the following significant features: (i) Paromomycin binding enhances the thermal stabilities of the RNA.RNA and DNA.RNA duplexes to similar extents, with this thermal enhancement being substantially greater in magnitude than that of the DNA.DNA duplex. (ii) Paromomycin binding to the DNA.RNA hybrid duplex induces CD changes consistent with a shift from an A-like to a more canonical A-conformation. (iii) Paromomycin binding to all three octamer duplexes is linked to the uptake of a similar number of protons, with the magnitude of this number being dependent on pH. (iv) The affinity of paromomycin for the three host duplexes follows the hierarchy, RNA.RNA > DNA.RNA >> DNA.DNA. (v) The observed affinity of paromomycin for the RNA.RNA and DNA.RNA duplexes decreases with increasing pH. (vi) The binding

of paromomycin to the DNA.RNA hybrid duplex inhibits both RNase H- and RNase A-mediated cleavage of the RNA strand. We discuss the implications of our combined results with regard to the specific targeting of DNA.RNA hybrid duplex domains and potential antiretroviral applications.

Boraston, A.B., Wang, D., and Burke, R.D. (2006) Blood Group Antigen Recognition by a Streptococcus pneumoniae Virulence Factor. *J. Biol. Chem* **281**, 35263-35271.

Abstract: The Streptococcus pneumoniae fucose utilization operon includes a gene encoding a virulence factor that belongs to family 98 in the glycoside hydrolase classification. This protein contains a C-terminal triplet of fucose binding modules that have significant amino acid sequence identity with the Anguilla anguilla fucoselectin. Functional studies of these fucose binding modules reveal binding to fucosylated oligosaccharides and suggest the importance of multivalent binding. The high resolution crystal structures of ligand bound forms of one fucose binding module uncovers the molecular basis of fucose, ABH blood group antigen, and Lewisy antigen binding. These studies are extended by fluorescence microscopy to show specific binding to mouse lung tissue. These modules define a new family of carbohydrate binding modules now classified as family 47.

Bridges K. G., Chow C. S., and Coen D. M. (2001) Identification of crucial hydrogen-bonding residues for the interaction of herpes simplex virus DNA polymerase subunits via peptide display, mutational, and calorimetric approaches. *J Virol* **75**, 4990-4998.

Abstract: The catalytic subunit, Pol, of herpes simplex virus DNA polymerase interacts via its extreme C terminus with the processivity subunit, UL42. This interaction is critical for viral replication and thus a potential target for antiviral drug action. To investigate the Pol-binding region on UL42, we engineered UL42 mutations but also used random peptide display to identify artificial ligands of the Pol C terminus. The latter approach selected ligands with homology to residues 171 to 176 of UL42. Substitution of glutamine 171 with alanine greatly impaired binding to Pol and stimulation of long-chain DNA synthesis by Pol, identifying this residue as crucial for subunit interactions. To study these interactions quantitatively, we used isothermal titration calorimetry and wild-type and mutant forms of Pol-derived peptides and UL42. Each of three peptides corresponding to either the last 36, 27, or 18 residues of Pol bound specifically to UL42 in a 1:1 complex with a dissociation constant of 1 to 2 μ M. Thus, the last 18 residues suffice for most of the binding energy, which was due mainly to a change in enthalpy. Substitutions at positions corresponding to Pol residue 1228 or 1229 or at UL42 residue 171 abolished or greatly reduced binding. These residues participate in hydrogen bonds observed in the crystal structure of the C terminus of Pol bound to UL42. Thus, interruption of these few bonds is sufficient to disrupt the interaction, suggesting that small molecules targeting the relevant side chains could interfere with Pol-UL42 binding.

Bullock A. N., Debreczeni J. E., Fedorov O. Y., Nelson A., Marsden B. D., and Knapp S. (2005) Structural basis of inhibitor specificity of the human protooncogene proviral insertion site in moloney murine leukemia virus (PIM-1) kinase. *J Med Chem* **48**, 7604-7614.

Abstract: The kinase PIM-1 plays a pivotal role in cytokine signaling and is implicated in the development of a number of tumors. The three-dimensional structure of PIM-1 is characterized by a unique hinge region which lacks a second hydrogen bond donor and makes it particularly important to determine how inhibitors bind to this kinase. We determined the structures of PIM-1 in complex with bisindolylmaleimide (BIM-1) and established the structure-activity relationship (SAR) for this inhibitor class. In addition, we screened a kinase targeted library and identified a number of high affinity inhibitors of PIM-1 such as imidazo[1,2-b]pyridazines, pyrazolo[1,5-a]pyrimidines, and members of the flavonoid family. In this paper we present an initial SAR of the identified scaffolds determined on the basis of a thermostability shift assay, calorimetric binding data, and biochemical assays which may find applications for the treatment of PIM-1 dependent cancer types.

Carneiro F. A., Bianconi M. L., Weissmuller G., Stauffer F., and Da Poian A. T. (2002) Membrane recognition by vesicular stomatitis virus involves enthalpy-driven protein-lipid interactions. *J Virol* **76**, 3756-3764.

Abstract: Vesicular stomatitis virus (VSV) infection depends on the fusion of viral and cellular membranes, which is mediated by virus spike glycoprotein G at the acidic environment of the endosomal compartment. VSV G protein does not contain a hydrophobic amino acid sequence similar to the fusion

peptides found among other viral glycoproteins, suggesting that membrane recognition occurs through an alternative mechanism. Here we studied the interaction between VSV G protein and liposomes of different phospholipid composition by force spectroscopy, isothermal titration calorimetry (ITC), and fluorescence spectroscopy. Force spectroscopy experiments revealed the requirement for negatively charged phospholipids for VSV binding to membranes, suggesting that this interaction is electrostatic in nature. In addition, ITC experiments showed that VSV binding to liposomes is an enthalpically driven process. Fluorescence data also showed the lack of VSV interaction with the vesicles as well as inhibition of VSV-induced membrane fusion at high ionic strength. Intrinsic fluorescence measurements showed that the extent of G protein conformational changes depends on the presence of phosphatidylserine (PS) on the target membrane. Although the increase in PS content did not change the binding profile, the rate of the fusion reaction was remarkably increased when the PS content was increased from 25 to 75%. On the basis of these data, we suggest that G protein binding to the target membrane essentially depends on electrostatic interactions, probably between positive charges on the protein surface and negatively charged phospholipids in the cellular membrane. In addition, the fusion is exothermic, indicating no entropic constraints to this process.

Carneiro F. A., Lapido-Loureiro P. A., Cordo S. M., Stauffer F., Weissmuller G., Bianconi M. L., Juliano M. A., Juliano L., Bisch P. M., and Poian A. T. (2006) Probing the interaction between vesicular stomatitis virus and phosphatidylserine. *Eur Biophys J* **35**, 145-154.

Abstract: The entry of enveloped animal viruses into their host cells always depends on membrane fusion triggered by conformational changes in viral envelope glycoproteins. Vesicular stomatitis virus (VSV) infection is mediated by virus spike glycoprotein G, which induces membrane fusion between the viral envelope and the endosomal membrane at the acidic environment of this compartment. In this work, we evaluated VSV interactions with membranes of different phospholipid compositions, at neutral and acidic pH, using atomic force microscopy (AFM) operating in the force spectroscopy mode, isothermal calorimetry (ITC) and molecular dynamics simulation. We found that the binding forces differed dramatically depending on the membrane phospholipid composition, revealing a high specificity of G protein binding to membranes containing phosphatidylserine (PS). In a previous work, we showed that the sequence corresponding amino acid 164 of VSV G protein was as efficient as the virus in catalyzing membrane fusion at pH 6.0. Here, we used this sequence to explore VSV-PS interaction using ITC. We found that peptide binding to membranes was exothermic, suggesting the participation of electrostatic interactions. Peptide-membrane interaction at pH 7.5 was shown to be specific to PS and dependent on the presence of His residues in the fusion peptide. The application of the simplified continuum Gouy-Chapman theory to our system predicted a pH of 5.0 at membrane surface, suggesting that the His residues should be protonated when located close to the membrane. Molecular dynamics simulations suggested that the peptide interacts with the lipid bilayer through its N-terminal residues, especially Val(145) and His(148).

Cervoni L., Lascu I., Xu Y., Gonin P., Morr M., Merouani M., Janin J., and Giartosio A. (2001) Binding of nucleotides to nucleoside diphosphate kinase: a calorimetric study. *Biochemistry* **40**, 4583-4589.

Abstract: The source of affinity for substrates of human nucleoside diphosphate (NDP) kinases is particularly important in that its knowledge could be used to design more effective antiviral nucleoside drugs (e.g., AZT). We carried out a microcalorimetric study of the binding of enzymes from two organisms to various nucleotides. Isothermal titration calorimetry has been used to characterize the binding in terms of ΔG degrees, ΔH degrees and ΔS degrees. Thermodynamic parameters of the interaction of ADP with the hexameric NDP kinase from *Dictyostelium discoideum* and with the tetrameric enzyme from *Myxococcus xanthus*, at 20 degrees C, were similar and, in both cases, binding was enthalpy-driven. The interactions of ADP, 2'-deoxyADP, GDP, and IDP with the eukaryotic enzyme differed in enthalpic and entropic terms, whereas the ΔG degrees values obtained were similar due to enthalpy--entropy compensation. The binding of the enzyme to nonphysiological nucleotides, such as AMP--PNP, 3'-deoxyADP, and 3'-deoxy-3'-amino-ADP, appears to differ in several respects. Crystallography of the protein bound to 3'-deoxy-3'-amino-ADP showed that the drug was in a distorted position, and was unable to interact correctly with active site side chains. The interaction of pyrimidine nucleoside diphosphates with the hexameric enzyme is characterized by a lower affinity than that with purine nucleotides. Titration showed the stoichiometry of the interaction to be abnormal, with 9--12 binding sites/hexamer. The presence of supplementary binding sites might have physiological implications.

Chen S., Chen L., Tan J., Chen J., Du L., Sun T., Shen J., Chen K., Jiang H., and Shen X. (2005) Severe acute respiratory syndrome coronavirus 3C-like proteinase N terminus is indispensable for proteolytic activity but not for enzyme dimerization. Biochemical and thermodynamic investigation in conjunction with molecular dynamics simulations. *J Biol Chem* **280**, 164-173.

Abstract: Severe acute respiratory syndrome (SARS) coronavirus is a novel human coronavirus and is responsible for SARS infection. SARS coronavirus 3C-like proteinase (SARS 3CL(pro)) plays key roles in viral replication and transcription and is an attractive target for anti-SARS drug discovery. In this report, we quantitatively characterized the dimerization features of the full-length and N-terminal residues 1-7 deleted SARS 3CL(pro)s by using glutaraldehyde cross-linking SDS-PAGE, size-exclusion chromatography, and isothermal titration calorimeter techniques. Glutaraldehyde cross-linking SDS-PAGE and size-exclusion chromatography results show that, similar to the full-length SARS 3CL(pro), the N-terminal deleted SARS 3CL(pro) still remains a dimer/monomer mixture within a wide range of protein concentrations. Isothermal titration calorimeter determinations indicate that the equilibrium dissociation constant ($K(d)$) of the N-terminal deleted proteinase dimer (262 microm) is very similar to that of the full-length proteinase dimer (227 microm). Enzymatic activity assay using the fluorescence resonance energy transfer method reveals that N-terminal deletion results in almost complete loss of enzymatic activity for SARS 3CL(pro). Molecular dynamics and docking simulations demonstrate the N-terminal deleted proteinase dimer adopts a state different from that of the full-length proteinase dimer, which increases the angle between the two protomers and reduces the binding pocket that is not beneficial to the substrate binding. This conclusion is verified by the surface plasmon resonance biosensor determination, indicating that the model substrate cannot bind to the N-terminal deleted proteinase. These results suggest the N terminus is not indispensable for the proteinase dimerization but may fix the dimer at the active state and is therefore vital to enzymatic activity.

Cheng A., Wong S. M. and Yuan Y. A. (2008) Structural basis for dsRNA recognition by NS1 protein of influenza A virus. *Cell Res (epublication)*.

Abstract: Influenza A viruses are important human pathogens causing periodic pandemic threats. Nonstructural protein 1 (NS1) protein of influenza A virus (NS1A) shields the virus against host defense. Here, we report the crystal structure of NS1A RNA-binding domain (RBD) bound to a double-stranded RNA (dsRNA) at 1.7Å. NS1A RBD forms a homodimer to recognize the major groove of A-form dsRNA in a length-independent mode by its conserved concave surface formed by dimeric anti-parallel alpha-helices. dsRNA is anchored by a pair of invariable arginines (Arg38) from both monomers by extensive hydrogen bonds. In accordance with the structural observation, isothermal titration calorimetry assay shows that the unique Arg38-Arg38 pair and two Arg35-Arg46 pairs are crucial for dsRNA binding, and that Ser42 and Thr49 are also important for dsRNA binding. Agrobacterium co-infiltration assay further supports that the unique Arg38 pair plays important roles in dsRNA binding in vivo. *Cell Research advance online publication 23 September 2008; doi: 10.1038/cr.2008.288*

Cicero D. O., Nadra A. D., Eliseo T., Dellarole M., Paci M., and Prat-Gay G. (2006) Structural and thermodynamic basis for the enhanced transcriptional control by the human papillomavirus strain-16 E2 protein. *Biochemistry* **45**, 6551-6560.

Abstract: Strain 16 of the human papillomavirus is responsible for the largest number of cases of cervical cancers linked to this virus, and the E2 protein is the transcriptional regulator of all viral genes. We present the first structure for the DNA binding domain of HPV16 E2 bound to DNA, and in particular, to a natural cognate sequence. The NMR structure of the protein backbone reveals that the overall conformation remains virtually unchanged, and chemical shift analysis of the protein bound to a shorter DNA duplex uncovered a contact out of the minimal E2 DNA binding site, made by lysine 349. This contact was confirmed by titration calorimetry and mutagenesis, with a contribution of 1.0 kcal mol⁻¹ to binding energy. HPV16 E2 has the highest DNA binding affinity and exerts a strict transcriptional control, translated into the repression of the E6 and E7 oncogenes. These novel features provide the structural and thermodynamic basis for this tight transcriptional control, the loss of which correlates with carcinogenesis.

Cole J. L. and Garsky V. M. (2001) Thermodynamics of peptide inhibitor binding to HIV-1 gp41. *Biochemistry* **40**, 5633-5641.

Abstract: The gp41 subunit of the human immunodeficiency virus type 1 envelope glycoprotein mediates fusion of the cellular and viral membranes. The gp41 ectodomain is a trimer of alpha-helical hairpins,

where N-terminal helices form a parallel three-stranded coiled-coil core and C-terminal helices pack around the core. A deep hydrophobic pocket on the N-terminal core represents an attractive target for antiviral therapeutics. We have employed a soluble derivative of the gp41 core ectodomain and small cyclic disulfide D-peptide inhibitors to define the stoichiometry, affinity, and thermodynamics of ligand binding to this pocket using isothermal titration calorimetry. These inhibitors bind with micromolar affinity to the pocket with the expected stoichiometry of three peptides per gp41 core trimer. There are no cooperative interactions among the three binding sites. Linear eight- or nine-residue D-peptides derived from the pocket-binding domain of the cyclic molecules also bind specifically. A negative heat capacity change is observed and is consistent with burial of hydrophobic surface upon binding. Contrary to expectations for a reaction dominated by the classical hydrophobic effect, peptide binding is enthalpically driven and is opposed by an unfavorable negative entropy change. The calorimetry data support models whereby dominant negative inhibitors bind to a transiently exposed surface on the prefusion intermediate state of gp41 and disrupt subsequent resolution to the fusion-active six-stranded hairpin conformation.

Cowan J. A., Ohyama T., Howard K., Rausch J. W., Cowan S. M., and Le Grice S. F. (2000) Metal-ion stoichiometry of the HIV-1 RT ribonuclease H domain: evidence for two mutually exclusive sites leads to new mechanistic insights on metal-mediated hydrolysis in nucleic acid biochemistry. *J Biol Inorg Chem* **5**, 67-74.

Abstract: Crystallographic studies of the Mn(2+)-doped RNase H domain of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) have revealed two bound Mn²⁺ separated by approximately 4Å and surrounded by a cluster of four conserved carboxylates. Escherichia coli RNase H is structurally similar to the RNase H domain of HIV-1 RT, but requires one divalent metal cation for its activity, implying either that the HIV-1 RT RNase H domain contrasts in its ability to bind two divalent metal ions, or that the crystallographic data reflect specific use of Mn²⁺ and/ or the doping technique employed. Metal binding stoichiometry has been determined for Mn²⁺ and the biologically more relevant Mg²⁺ cation by solution calorimetric studies of native and recombinant p66/p51 HIV-1 RT. Three Mn²⁺ ions bind to HIV-1 RT apo-enzyme: one at the DNA polymerase and two at the RNase H catalytic center, the latter being consistent with crystallographic results. However, only one Mg²⁺ ion is bound in the RNase H catalytic center. Several mechanistic implications arise from these results, including the possibility of mutually exclusive Mg²⁺ binding sites that might be occupied according to the specific reaction being catalyzed by the multifunctional RNase H domain. The occurrence of distinct binding stoichiometries for Mg²⁺ and Mn²⁺ to multifunctional enzymes has previously been reported.

D'Aquino J. A., Tetenbaum-Novatt J., White A., Berkovitch F., and Ringe D. (2005) Mechanism of metal ion activation of the diphtheria toxin repressor DtxR. *Proc Natl Acad Sci U S A*, **102**, 18408-13.

Abstract: The diphtheria toxin repressor (DtxR) is a metal ion-activated transcriptional regulator that has been linked to the virulence of *Corynebacterium diphtheriae*. Structure determination has shown that there are two metal ion binding sites per repressor monomer, and site-directed mutagenesis has demonstrated that binding site 2 (primary) is essential for recognition of the target DNA repressor, leaving the role of binding site 1 (ancillary) unclear. Calorimetric techniques have demonstrated that although binding site 1 (ancillary) has high affinity for metal ion with a binding constant of 2×10^7 , binding site 2 (primary) is a low-affinity binding site with a binding constant of 6.3×10^4 . These two binding sites act in an independent fashion, and their contribution can be easily dissected by traditional mutational analysis. Our results clearly demonstrate that binding site 1 (ancillary) is the first one to be occupied during metal ion activation, playing a critical role in stabilization of the repressor. In addition, structural data obtained for the mutants Ni-DtxR(H79A,C102D), reported here, and the previously reported DtxR(H79A) have allowed us to propose a mechanism of metal activation for DtxR.

Da Poian A.T., Carneiro F.A., and Stauffer F. (2005) Viral membrane fusion: is glycoprotein G of rhabdoviruses a representative of a new class of viral fusion proteins? *Braz J Med Biol Res.* **38**, 813-23.
Abstract: Enveloped viruses always gain entry into the cytoplasm by fusion of their lipid envelope with a cell membrane. Some enveloped viruses fuse directly with the host cell plasma membrane after virus binding to the cell receptor. Other enveloped viruses enter the cells by the endocytic pathway, and fusion depends on the acidification of the endosomal compartment. In both cases, virus-induced membrane fusion is triggered by conformational changes in viral envelope glycoproteins. Two different classes of viral fusion proteins have been described on the basis of their molecular architecture. Several structural data

permitted the elucidation of the mechanisms of membrane fusion mediated by class I and class II fusion proteins. In this article, we review a number of results obtained by our laboratory and by others that suggest that the mechanisms involved in rhabdovirus fusion are different from those used by the two well-studied classes of viral glycoproteins. We focus our discussion on the electrostatic nature of virus binding and interaction with membranes, especially through phosphatidylserine, and on the reversibility of the conformational changes of the rhabdovirus glycoprotein involved in fusion. Taken together, these data suggest the existence of a third class of fusion proteins and support the idea that new insights should emerge from studies of membrane fusion mediated by the G protein of rhabdoviruses. In particular, the elucidation of the three-dimensional structure of the G protein or even of the fusion peptide at different pH's might provide valuable information for understanding the fusion mechanism of this new class of fusion proteins.

Dellarole M., Sanchez I. E., Freire E. and de Prat-Gay G. (2007) Increased stability and DNA site discrimination of "single chain" variants of the dimeric beta-barrel DNA binding domain of the human papillomavirus E2 transcriptional regulator. *Biochemistry* **46**, 12441-12450.

Abstract: Human papillomavirus infects millions of people worldwide and is a causal agent of cervical cancer in women. The HPV E2 protein controls the expression of all viral genes through binding of its dimeric C-terminal domain (E2C) to its target DNA site. We engineered monomeric versions of the HPV16 E2C, in order to probe the link of the dimeric beta-barrel fold to stability, dimerization, and DNA binding. Two single-chain variants, with 6 and 12 residue linkers (scE2C-6 and scE2C-12), were purified and characterized. Spectroscopy and crystallography show that the native structure is unperturbed in scE2C-12. The single chain variants are stabilized with respect to E2C, with effective concentrations of 0.6 to 6 mM. The early folding events of the E2C dimer and scE2C-12 are very similar and include formation of a compact species in the submillisecond time scale and a non-native monomeric intermediate with a half-life of 25 ms. However, monomerization changes the unfolding mechanism of the linked species from two-state to three-state, with a high-energy intermediate. Binding to the specific target site is up to 5-fold tighter in the single chain variants. Nonspecific DNA binding is up to 7-fold weaker in the single chain variants, leading to an overall 10-fold increased site discrimination capacity, the largest described so far for linked DNA binding domains. Titration calorimetric binding analysis, however, shows almost identical behavior for dimer and single-chain species, suggesting very subtle changes behind the increased specificity. Global analysis of the mechanisms probed suggests that the dynamics of the E2C domain, rather than the structure, are responsible for the differential properties. Thus, the plastic and dimeric nature of the domain did not evolve for a maximum affinity, specificity, and stability of the quaternary structure, likely because of regulatory reasons and for roles other than DNA binding played by partly folded dimeric or monomeric conformers.

D'Souza V., Melamed J., Habib D., Pullen K., Wallace K., and Summers M. F. (2001) Identification of a high affinity nucleocapsid protein binding element within the Moloney murine leukemia virus Psi-RNA packaging signal: implications for genome recognition. *J Mol Biol* **314**, 217-232.

Abstract: Murine leukemia virus (MLV) is currently the most widely used gene delivery system in gene therapy trials. The simple retrovirus packages two copies of its RNA genome by a mechanism that involves interactions between the nucleocapsid (NC) domain of a virally-encoded Gag polyprotein and a segment of the RNA genome located just upstream of the Gag initiation codon, known as the Psi-site. Previous studies indicated that the MLV Psi-site contains three stem loops (SLB-SLD), and that stem loops SLC and SLD play prominent roles in packaging. We have developed a method for the preparation and purification of large quantities of recombinant Moloney MLV NC protein, and have studied its interactions with a series of oligoribonucleotides that contain one or more of the Psi-RNA stem loops. At RNA concentrations above approximately 0.3 mM, isolated stem loop SLB forms a duplex and stem loops SL-C and SL-D form kissing complexes, as expected from previous studies. However, neither the monomeric nor the dimeric forms of these isolated stem loops binds NC with significant affinity. Longer constructs containing two stem loops (SL-BC and SL-CD) also exhibit low affinities for NC. However, NC binds with high affinity and stoichiometrically to both the monomeric and dimeric forms of an RNA construct that contains all three stem loops (SL-BCD; $K_d=132(+/-55)$ nM). Titration of SL-BCD with NC also shifts monomer-dimer equilibrium toward the dimer. Mutagenesis experiments demonstrate that the conserved GACG tetraloops of stem loops C and D do not influence the monomer-dimer equilibrium of SL-BCD, that the tetraloop of

stem loop B does not participate directly in NC binding, and that the tetraloops of stem loops C and D probably also do not bind to NC. These surprising results differ considerably from those observed for HIV-1, where NC binds to individual stem loops with high affinity via interactions with exposed residues of the tetraloops. The present results indicate that MLV NC binds to a pocket or surface that only exists in the presence of all three stem loops.

Egloff, M.P., Malet, H., Putics, A., Heinonen, M., Dutartre H., Frangeul, A., Gruez, A., Campanacci, V., Cambillau, C., Ziebuhr, J., Ahola, T., and Canard, B. (2006) Structural and Functional Basis for ADP-Ribose and Poly(ADP-Ribose) Binding by Viral Macro Domains. *J Virol*, **80**, 8493-8502.

Abstract: Macro domains constitute a protein module family found associated with specific histones and proteins involved in chromatin metabolism. In addition, a small number of animal RNA viruses, such as corona- and toroviruses, alphaviruses, and hepatitis E virus, encode macro domains for which, however, structural and functional information is extremely limited. Here, we characterized the macro domains from hepatitis E virus, Semliki Forest virus, and severe acute respiratory syndrome coronavirus (SARS-CoV). The crystal structure of the SARS-CoV macro domain was determined at 1.8-Å resolution in complex with ADP-ribose. Information derived from structural, mutational, and sequence analyses suggests a close phylogenetic and, most probably, functional relationship between viral and cellular macro domain homologs. The data revealed that viral macro domains have relatively poor ADP-ribose 1'-phosphohydrolase activities (which were previously proposed to be their biologically relevant function) but bind efficiently free and poly(ADP-ribose) polymerase 1-bound poly(ADP-ribose) in vitro. Collectively, these results suggest to further evaluate the role of viral macro domains in host response to viral infection.

Filikov A. V. and James T. L. (1998) Structure-based design of ligands for protein basic domains: application to the HIV-1 Tat protein. *J Comput Aided Mol Des* **12**, 229-240.

Abstract: A methodology has been developed for designing ligands to bind a flexible basic protein domain where the structure of the domain is essentially known. It is based on an empirical binding free energy function developed for highly charged complexes and on Monte Carlo simulations in internal coordinates with both the ligand and the receptor being flexible. HIV-1 encodes a transactivating regulatory protein called Tat. Binding of the basic domain of Tat to TAR RNA is required for efficient transcription of the viral genome. The structure of a biologically active peptide containing the Tat basic RNA-binding domain is available from NMR studies. The goal of the current project is to design a ligand which will bind to that basic domain and potentially inhibit the TAR-Tat interaction. The basic domain contains six arginine and two lysine residues. Our strategy was to design a ligand for arginine first and then a superligand for the basic domain by joining arginine ligands with a linker. Several possible arginine ligands were obtained by searching the Available Chemicals Directory with DOCK 3.5 software. Phytic acid, which can potentially bind multiple arginines, was chosen as a building block for the superligand. Calorimetric binding studies of several compounds to methylguanidine and Arg-/Lys-containing peptides were performed. The data were used to develop an empirical binding free energy function for prediction of affinity of the ligands for the Tat basic domain. Modeling of the conformations of the complexes with both the superligand and the basic domain being flexible has been carried out via Biased Probability Monte Carlo (BPMC) simulations in internal coordinates (ICM 2.6 suite of programs). The simulations used parameters to ensure correct folding, i.e., consistent with the experimental NMR structure of a 25-residue Tat peptide, from a random starting conformation. Superligands for the basic domain were designed by joining together two molecules of phytic acid with peptidic and peptidomimetic linkers. The linkers were refined by varying the length and side chains of the linking residues, carrying out BPMC simulations, and evaluation of the binding free energy for the best energy conformation. The dissociation constant of the best ligand designed is estimated to be in the low- to mid-nanomolar range.

Fisher R. J., Fivash M. J., Stephen A. G., Hagan N. A., Shenoy S. R., Medaglia M. V., Smith L. R., Worthy K. M., Simpson J. T., Shoemaker R., McNitt K. L., Johnson D. G., Hixson C. V., Gorelick R. J., Fabris D., Henderson L. E., and Rein A. (2006) Complex interactions of HIV-1 nucleocapsid protein with oligonucleotides. *Nucleic Acids Res* **34**, 472-484.

Abstract: The HIV-1 nucleocapsid (NC) protein is a small, basic protein containing two retroviral zinc fingers. It is a highly active nucleic acid chaperone; because of this activity, it plays a crucial role in virus replication as a cofactor during reverse transcription, and is probably important in other steps of the

replication cycle as well. We previously reported that NC binds with high-affinity to the repeating sequence d(TG)_n. We have now analyzed the interaction between NC and d(TG)₄ in considerable detail, using surface plasmon resonance (SPR), tryptophan fluorescence quenching (TFQ), fluorescence anisotropy (FA), isothermal titration calorimetry (ITC) and electrospray ionization Fourier transform mass spectrometry (ESI-FTMS). Our results show that the interactions between these two molecules are surprisingly complex: while the K(d) for binding of a single d(TG)₄ molecule to NC is only approximately 5 nM in 150 mM NaCl, a single NC molecule is capable of interacting with more than one d(TG)₄ molecule, and conversely, more than one NC molecule can bind to a single d(TG)₄ molecule. The strengths of these additional binding reactions are quantitated. The implications of this multivalency for the functions of NC in virus replication are discussed.

Garzon M. T., Lidon-Moya M. C., Barrera F. N., Prieto A., Gomez J., Mateu M. G., and Neira J. L. (2004) The dimerization domain of the HIV-1 capsid protein binds a capsid protein-derived peptide: a biophysical characterization. *Protein Sci* **13**, 1512-1523.

Abstract: The type 1 HIV presents a conical capsid formed by approximately 1500 units of the capsid protein, CA. Homodimerization of CA via its C-terminal domain, CA-C, constitutes a key step in virion assembly. CA-C dimerization is largely mediated by reciprocal interactions between residues of its second alpha-helix. Here, we show that an N-terminal-acetylated and C-terminal-amidated peptide, CAC1, comprising the sequence of the CA-C dimerization helix plus three flanking residues at each side, is able to form a complex with the entire CA-C domain. Thermal denaturation measurements followed by circular dichroism (CD), NMR, and size-exclusion chromatography provided evidence of the interaction between CAC1 and CA-C. The apparent dissociation constant of the heterocomplex formed by CA-C and CAC1 was determined by several biophysical techniques, namely, fluorescence (using an anthraniloyl-labeled peptide), affinity chromatography, and isothermal titration calorimetry. The three techniques yielded similar values for the apparent dissociation constant, in the order of 50 μM. This apparent dissociation constant was only five times higher than was the dissociation constant of both CA-C and the intact capsid protein homodimers (10 μM).

Gomez J. and Freire E. (1995) Thermodynamic mapping of the inhibitor site of the aspartic protease endothiapepsin. *J Mol Biol* **252**, 337-350.

Abstract: The discovery that the protease from the human immunodeficiency virus (HIV) belongs to the aspartic protease family has generated renewed interest in this class of proteins. In this paper, the interactions of endothiapepsin, an aspartic proteinase from the fungus *Endothia parasitica*, with the inhibitor pepstatin A have been studied by high-sensitivity calorimetric techniques. These experiments have permitted a complete characterization of the temperature and pH-dependence of the binding energetics. The binding reaction is characterized by negative intrinsic binding enthalpy and negative heat capacity changes. The association constant is maximal at low pH ($2 \times 10^9 \text{ M}^{-1}$ at pH 3) but decreases upon increasing pH ($8.1 \times 10^6 \text{ M}^{-1}$ at pH 7). The binding of the inhibitor is coupled to the protonation of one of the aspartic moieties in the Asp dyad of the catalytic site of the protein. This phenomenon is responsible for the decrease in the apparent affinity of the inhibitor for the enzyme upon increasing pH. The experimental results presented here indicate that the binding of the inhibitor is favored both enthalpically and entropically. While the favorable enthalpic contribution is intuitively expected, the favorable entropic contribution is due to the large gain in solvent-related entropy associated with the burial of a large hydrophobic surface, that overcompensates the loss in conformational and translational/rotational degrees of freedom upon complex formation. The characteristics of the molecular recognition process have been evaluated by means of structure-based thermodynamic analysis. Three regions in the protein contribute significantly to the free energy of binding: the residues surrounding the Asp dyad (Asp32 in the N-terminal lobe and Asp215 in the C-terminal domain) and the flap region (Ile73 to Asp77). In addition, the rearrangement of residues that are not in immediate contact with the inhibitor provides close to 40% of the protease contribution to the binding free energy. On the other hand, the two statine residues provide more than half of the inhibitor contributions to the total free energy of binding. It is demonstrated that a previously developed empirical structural parametrization of the thermodynamic parameters that define the Gibbs energy, accurately accounts for the binding energetics and its temperature and pH-dependence.

Groft C. M. and Burley S. K. (2002) Recognition of eIF4G by rotavirus NSP3 reveals a basis for mRNA circularization. *Mol Cell* **9**, 1273-1283.

Abstract: Rotaviruses, segmented double-stranded RNA viruses, co-opt the eukaryotic translation machinery with the aid of nonstructural protein 3 (NSP3), a rotaviral functional homolog of the cellular poly(A) binding protein (PABP). NSP3 binds to viral mRNA 3' consensus sequences and circularizes mRNA via interactions with eIF4G. Here, we present the X-ray structure of the C-terminal domain of NSP3 (NSP3-C) recognizing a fragment of eIF4G. Homodimerization of NSP3-C yields a symmetric, elongated, largely alpha-helical structure with two hydrophobic eIF4G binding pockets at the dimer interface. Site-directed mutagenesis and isothermal titration calorimetry documented that NSP3 and PABP use analogous eIF4G recognition strategies, despite marked differences in tertiary structure.

Harris S. L. and Fernsten P. (2008) Thermodynamics and Binding Density of Binding of a Panel of Anti-Polysaccharide Antibodies to High Molecular Weight Capsular Polysaccharides. *Clin. Vaccine Immunol* (*publication*).

Abstract: The interaction between anti-polysaccharide (PS) antibodies and their antigens was investigated via isothermal titration calorimetry (ITC) to determine the thermodynamic binding constant (K), the enthalpy of binding (ΔH) and the binding density (N) to high molecular weight PS. From these values, the entropy of binding (ΔS) was calculated. The thermodynamic parameters of binding to high molecular weight capsular PS are reported for two monoclonal antibodies (mAbs) with different specificities for meningococcal Serogroup C PS, five mAbs specific for different pneumococcal serotypes, and Fab fragments of two anti-pneumococcal mAbs. The K values were in the 10^6 to 10^7 M⁻¹ range, which are one to two orders of magnitude greater than previously reported K values derived from antibody-oligosaccharide interactions. The enthalpy change, ΔH , associated with binding was favorable for each mAb and Fab fragment. The entropy change, ΔS , associated with binding was also generally favorable for both the mAbs and Fab fragments, with the exception of the anti-serotype 14 mAb and its Fab fragment. N provides information regarding how densely mAbs or Fabs can bind along PS chains and, expressed in terms of monosaccharides, was very similar for the seven mAbs, with an average of twelve monosaccharides per bound mAb. The N for each Fab was smaller, with five or seven monosaccharides per bound Fab. These results suggest that steric interactions between antibody molecules are a major influence on N or binding density of high affinity mAbs to capsular PS

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.

Heilman-Miller S. L., Wu T., and Levin J. G. (2004) Alteration of nucleic acid structure and stability modulates the efficiency of minus-strand transfer mediated by the HIV-1 nucleocapsid protein. *J Biol Chem* **279**, 44154-44165.

Abstract: During human immunodeficiency virus type 1 minus-strand transfer, the nucleocapsid protein (NC) facilitates annealing of the complementary repeat regions at the 3'-ends of acceptor RNA and minus-

strand strong-stop DNA ((-) SSDNA). In addition, NC destabilizes the highly structured complementary trans-activation response element (TAR) stem-loop (TAR DNA) at the 3'-end of (-) SSDNA and inhibits TAR-induced self-priming, a dead-end reaction that competes with minus-strand transfer. To investigate the relationship between nucleic acid secondary structure and NC function, a series of truncated (-) SSDNA and acceptor RNA constructs were used to assay minus-strand transfer and self-priming in vitro. The results were correlated with extensive enzymatic probing and mFold analysis. As the length of (-) SSDNA was decreased, self-priming increased and was highest when the DNA contained little more than TAR DNA, even if NC and acceptor were both present; in contrast, truncations within TAR DNA led to a striking reduction or elimination of self-priming. However, destabilization of TAR DNA was not sufficient for successful strand transfer: the stability of acceptor RNA was also crucial, and little or no strand transfer occurred if the RNA was highly stable. Significantly, NC may not be required for in vitro strand transfer if (-) SSDNA and acceptor RNA are small, relatively unstructured molecules with low thermodynamic stabilities. Collectively, these findings demonstrate that for efficient NC-mediated minus-strand transfer, a delicate thermodynamic balance between the RNA and DNA reactants must be maintained.

Hoog S. S., Towler E. M., Zhao B., Doyle M. L., Debouck C., and Abdel-Meguid S. S. (1996) Human immunodeficiency virus protease ligand specificity conferred by residues outside of the active site cavity. *Biochemistry* **35**, 10279-10286.

Abstract: To gain greater understanding of the structural basis of human immunodeficiency virus (HIV) protease ligand specificity, we have crystallized and determined the structures of the HIV-1 protease (Val32Ile, Ile47Val, Val82Ile) triple mutant and simian immunodeficiency virus (SIV) protease in complex with SB203386, a tripeptide analogue inhibitor containing a C-terminal imidazole substituent as an amide bond isostere. SB203386 is a potent inhibitor of HIV-1 protease ($K_i = 18$ nM) but shows decreased inhibition of the HIV-1 protease (Val32Ile, Ile47Val, Val82Ile) triple mutant ($K_i = 112$ nM) and SIV protease ($K_i = 960$ nM). Although SB203386 binds in the active site cavity of the triple mutant in a similar fashion to its binding to the wild-type HIV-1 protease [Abdel-Meguid et al. (1994) *Biochemistry* **33**, 11671], it binds to SIV protease in an unexpected mode showing two inhibitor molecules each binding to half of the active site. Comparison of these two structures and that of the wild-type HIV-1 protease bound to SB203386 reveals that HIV protease ligand specificity is imparted by residues outside of the catalytic pocket, which causes subtle changes in its shape. Furthermore, this work illustrates the importance of structural studies in order to understand the structure-activity relationship (SAR) between related enzymes.

Houbaviy H. B. and Burley S. K. (2001) Thermodynamic analysis of the interaction between YY1 and the AAV P5 promoter initiator element. *Chem Biol* **8**, 179-187.

Abstract: BACKGROUND: We previously determined the co-crystal structure of the zinc finger region of transcription factor YY1 (YY1 Δ) bound to the initiator element (Inr) of the adenoassociated virus (AAV) P5 gene promoter [Houbaviy, H.B. et al. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13577-13582]. Our structure explained both binding specificity and the ability of YY1 to support specific, unidirectional transcription initiation. RESULTS: To further understand Inr recognition by YY1, we analyzed the YY1 Δ -Inr interaction by isothermal titration calorimetry (ITC) and used limited proteolysis, DNase I footprinting and missing nucleoside experiments to show that YY1 Δ and full-length YY1 (YY1WT) have indistinguishable DNA binding properties. CONCLUSIONS: YY1 binding occurs at an equilibrium dissociation constant (Kd) of about 1 μ M, and exhibits a large negative heat capacity change (ΔC_p). We analyzed the thermodynamic behavior of YY1 Δ in terms of buried solvent-accessible surface area resulting from interaction of two rigid bodies, which could not explain our measured value of ΔC_p . We must, therefore, postulate conformational changes in YY1 and/or the Inr or question the validity of current $\Delta C(p)$ analysis methods for protein-DNA interactions.

Jenkins H. T., Mark L., Ball G., Persson J., Lindahl G., Uhrin D., Blom A. M., and Barlow P. N. (2006) Human C4b-binding protein, structural basis for interaction with streptococcal M protein, a major bacterial virulence factor. *J Biol Chem* **281**, 3690-3697.

Abstract: Human C4b-binding protein (C4BP) protects host tissue, and those pathogens able to hijack this plasma glycoprotein, from complement-mediated destruction. We now show that the first two complement control protein (CCP) modules of the C4BP alpha-chain, plus the four residues connecting them, are necessary and sufficient for binding a bacterial virulence factor, the *Streptococcus pyogenes* M4 (Arp4) protein. Structure determination by NMR reveals two tightly coupled CCP modules in an elongated

arrangement within this region of C4BP. Chemical shift perturbation studies demonstrate that the N-terminal, hypervariable region of M4 binds to a site including strand 1 of CCP module 2. This interaction is accompanied by an intermodular reorientation within C4BP. We thus provide a detailed picture of an interaction whereby a pathogen evades complement.

Keller M., Tagawa T., Preuss M., and Miller A. D. (2002) Biophysical characterization of the DNA binding and condensing properties of adenoviral core peptide μ . *Biochemistry* **41**, 652-659.

Abstract: Cationic peptides containing Lys and Arg residues interact with DNA via charge-charge interactions and are known to play an important role in DNA charge neutralization and condensation processes. In this paper, we describe investigations of the interaction of the cationic adenovirus core complex peptide μ with a dodecameric ODN (12 bp) and pDNA (7528 bp) using a combination of fluorescence spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, and photon correlation spectroscopy. Comparisons are made with protamine, a cationic peptide well-known for DNA charge neutralization and condensation. Equilibrium dissociation constants are derived independently by both CD and ITC methods for the interaction between protamine or μ with pDNA ($K_d = 0.6\text{-}1 \mu\text{M}$). Thermodynamic data are also obtained by ITC, indicating strong charge-charge interactions. The interaction of protamine with pDNA takes place with decreasing entropy ($-28.7 \text{ cal mol}^{-1} \text{ K}^{-1}$); unusually, the interaction of μ with pDNA takes place with increasing entropy ($\Delta S \text{ degrees (bind)} = 11.3 \text{ cal mol}^{-1} \text{ K}^{-1}$). Although protamine and μ appear to destabilize pDNA double helix character to similar extents, according to CD thermal titration analyses, PCS studies show that interactions between μ and pDNA result in the formation of significantly more size-stable condensed particles than protamine. The enhanced flexibility and size stability of μ -DNA (MD) particles (80-110 nm) compared to protamine counterparts suggest that MD particles are ideal for use as a part of new nonviral gene delivery vectors.

Kim I., Liu C. W., and Puglisi J. D. (2006) Specific recognition of HIV TAR RNA by the dsRNA binding domains (dsRBD1-dsRBD2) of PKR. *J Mol Biol* **358**, 430-442.

Abstract: PKR (double-stranded RNA-dependent protein kinase) is an important component of host defense to virus infection. Binding of dsRNA to two dsRBDs (double-stranded RNA binding domains) of PKR modulates its own kinase activation. How structural features of natural target RNAs, such as bulges and loops, have an effect on the binding to two dsRBDs of PKR still remains unclear. By using ITC and NMR, we show here that both the bulge and loop of TAR RNA are necessary for the high affinity binding to dsRBD1-dsRBD2 of PKR with 1:1 stoichiometry. The binding site for the dsRBD1-dsRBD2 spans from upper bulge to lower stem of the TAR RNA, based on chemical shift mapping. The backbone resonances in the 40 kDa TAR.dsRBD1-dsRBD2 were assigned. NMR chemical shift perturbation data suggest that the beta1-beta2 loop of the dsRBD1 interacts with the TAR RNA, whereas that of the dsRBD2 is less involved in the TAR RNA recognition. In addition, the residues of the interdomain linker between the dsRBD1 and the dsRBD2 also show large chemical perturbations indicating that the linker is involved in the recognition of TAR RNA. The results presented here provide the biophysical and spectroscopic basis for high-resolution structural studies, and show how local RNA structural features modulate recognition by dsRBDs.

King N. M., Prabu-Jeyabalan M., Nalivaika E. A., Wigerinck P., de Bethune M. P., and Schiffer C. A. (2004) Structural and thermodynamic basis for the binding of TMC114, a next-generation human immunodeficiency virus type 1 protease inhibitor. *J Virol* **78**, 12012-12021.

Abstract: TMC114, a newly designed human immunodeficiency virus type 1 (HIV-1) protease inhibitor, is extremely potent against both wild-type (wt) and multidrug-resistant (MDR) viruses in vitro as well as in vivo. Although chemically similar to amprenavir (APV), the potency of TMC114 is substantially greater. To examine the basis for this potency, we solved crystal structures of TMC114 complexed with wt HIV-1 protease and TMC114 and APV complexed with an MDR (L63P, V82T, and I84V) protease variant. In addition, we determined the corresponding binding thermodynamics by isothermal titration calorimetry. TMC114 binds approximately 2 orders of magnitude more tightly to the wt enzyme ($K_d = 4.5 \times 10^{-12} \text{ M}$) than APV ($K_d = 3.9 \times 10^{-10} \text{ M}$). Our X-ray data (resolution ranging from 2.2 to 1.2 Å) reveal strong interactions between the bis-tetrahydrofuranyl urethane moiety of TMC114 and main-chain atoms of D29 and D30. These interactions appear largely responsible for TMC114's very favorable binding enthalpy to the wt protease (-12.1 kcal/mol). However, TMC114 binding to the MDR HIV-1 protease is reduced by a factor of 13.3, whereas the APV binding constant is reduced only by a factor of 5.1. However, even with the reduction in binding affinity to the MDR HIV protease, TMC114 still binds with an affinity that is more

than 1.5 orders of magnitude tighter than the first-generation inhibitors. Both APV and TMC114 fit predominantly within the substrate envelope, a property that may be associated with decreased susceptibility to drug-resistant mutations relative to that of first-generation inhibitors. Overall, TMC114's potency against MDR viruses is likely a combination of its extremely high affinity and close fit within the substrate envelope.

Kingston R. L., Baase W. A., and Gay L. S. (2004) Characterization of nucleocapsid binding by the measles virus and mumps virus phosphoproteins. *J Virol* **78**, 8630-8640.

Abstract: We report an analysis of the interaction between the P protein and the RNA-associated N protein (N-RNA) for both measles and mumps viruses with proteins produced in a bacterial expression system. During this study, we verified that the C-terminal tail of the N protein is not required for nucleocapsid formation. For both measles and mumps virus N, truncated proteins encompassing amino acids 1 to 375 assemble into nucleocapsid-like particles within the bacterial cell. For measles virus N, the binding site for the P protein maps to residues 477 to 505 within the tail of the molecule, a sequence relatively conserved among the morbilliviruses. For mumps virus N, a binding site for the P protein maps to the assembly domain of N (residues 1 to 398), while no strong binding of the P protein to the tail of N was detected. These results suggest that the site of attachment for the polymerase varies among the paramyxoviruses. Pulldown experiments demonstrate that the last 50 amino acids of both measles virus and mumps virus P (measles virus P, 457 to 507; mumps virus P, 343 to 391) by themselves constitute the nucleocapsid-binding domain (NBD). Spectroscopic studies show that the NBD is predominantly alpha-helical in both viruses. However, only in measles virus P is the NBD stable and folded, having a lesser degree of tertiary organization in mumps virus P. With isothermal titration calorimetry, we demonstrate that the measles virus P NBD binds to residues 477 to 505 of measles virus N with 1:1 stoichiometry. The dissociation constant (K_d) was determined to be 13 μ M at 20 degrees C and 35 μ M at 37 degrees C. Our data are consistent with a model in which an alpha-helical nucleocapsid binding domain, located at the C terminus of P, is responsible for tethering the viral polymerase to its template yet also suggest that, in detail, polymerase binding in morbilliviruses and rubulaviruses differs significantly.

Kraschnefski M. J., Bugarcic A., Fleming F. E., Yu X., von I. M., Coulson B. S. and Blanchard H. (2008) Effects on sialic acid recognition of amino acid mutations in the carbohydrate-binding cleft of the rotavirus spike protein. *Glycobiology*. (epublication)

Abstract: The rotavirus spike protein VP4 mediates attachment to host cells and subsequent membrane penetration. The VP8* domain of VP4 forms the spike tips and is proposed to recognise host cell surface glycans. For sialidase-sensitive rotaviruses such as rhesus (RRV) this recognition involves terminal sialic acids. We show here that RRV VP8*(64-224) protein competes with RRV infection of host cells, demonstrating its relevance to infection. In addition, we observe that the amino acids revealed by X-ray crystallography to be in direct contact with the bound sialic acid derivative methyl alpha-D-N-acetylneuraminide, and that are highly conserved amongst sialidase-sensitive rotaviruses, are residues that are also important in interactions with host-cell carbohydrates. Residues Arg101 and Ser190 of the RRV VP8* carbohydrate-binding site were mutated to assess their importance for binding to the sialic acid derivative and their competition with RRV infection of host cells. The crystallographic structure of the Arg(101)Ala mutant crystallised in the presence of sialic acid derivative was determined at 295 K to a resolution of 1.9 Å. Our multi-disciplinary study using X-ray crystallography, Saturation Transfer Difference nuclear magnetic resonance spectroscopy, isothermal titration calorimetry and competitive virus infectivity assays to investigate RRV wild-type and mutant VP8* proteins has provided the first evidence that the carbohydrate-binding cavity in RRV VP8* is used for host cell recognition, and this interaction is not only with the sialic acid portion but also other parts of the glycan structure

Krell T., Greco F., Nicolai M. C., Dubayle J., Renaud-Mongenien G., Poisson N., and Bernard I. (2003) The use of microcalorimetry to characterize tetanus neurotoxin, pertussis toxin and filamentous haemagglutinin. *Biotechnol Appl Biochem* **38**, 241-251.

Abstract: Tetanus neurotoxin (TeNT), pertussis toxin (PT) and pertussis filamentous haemagglutinin (FHA) are major virulence factors of *Clostridium tetani* and *Bordetella pertussis*, which are the causative agents of tetanus and whooping cough respectively. Inactivated forms of these virulence factors are the protein components of vaccines against these diseases. Here we report microcalorimetric studies to characterize these proteins. The microcalorimetric titration curves of TeNT with micelles of gangliosides

G(D1b), G(T1b) and G(Q1b) were biphasic. For these gangliosides a high-affinity binding site (K_D 45-277 nM) can be distinguished from a lower-affinity binding event (K_D 666-1190 nM). This is direct evidence for multiple binding sites for gangliosides of the (1b) series at TeNT as proposed by Emsley et al. [Emsley, Fotinou, Black, Fairweather, Charles, Watts, Hewitt and Isaacs (2000) *J. Biol. Chem.* 275, 8889-8894]. In agreement with previous reports, no binding was observed for gangliosides G(M1), G(M2), G(M3) and G(D2). The thermal denaturation of TeNT was characterized by two unfolding transitions centred around 57.4 and 62.4 degrees C. The conversion of TeNT into the toxoid form by formaldehyde treatment was accompanied by a large increase in T_m (the midpoint of protein unfolding transition, that is, the temperature at which half the protein is denatured and the other half is still present in its native form). Fetuin and asialofetuin bound to PT with similar affinities (K_D 420 and 335 nM respectively). Binding was largely enthalpy-driven and counterbalanced by an unfavourable entropy change, indicating a loss of conformational flexibility. The latter could account for the observed inhibition of ATP binding after binding to fetuin. Furthermore, the molecular limits of mature PT subunit S5 were defined by MS and N-terminal peptide sequencing. The differential-scanning-calorimetry thermogram of FHA shows four well-resolved unfolding transitions, a finding consistent with the sequential denaturation of four structural domains.

Kwong P. D., Doyle M. L., Casper D. J., Cicala C., Leavitt S. A., Majeed S., Steenbeke T. D., Venturi M., Chaiken I., Fung M., Katinger H., Parren P. W., Robinson J., Van Ryk D., Wang L., Burton D. R., Freire E., Wyatt R., Sodroski J., Hendrickson W. A., and Arthos J. (2002) HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* **420**, 678-682.

Abstract: The ability of human immunodeficiency virus (HIV-1) to persist and cause AIDS is dependent on its avoidance of antibody-mediated neutralization. The virus elicits abundant, envelope-directed antibodies that have little neutralization capacity. This lack of neutralization is paradoxical, given the functional conservation and exposure of receptor-binding sites on the gp120 envelope glycoprotein, which are larger than the typical antibody footprint and should therefore be accessible for antibody binding. Because gp120-receptor interactions involve conformational reorganization, we measured the entropies of binding for 20 gp120-reactive antibodies. Here we show that recognition by receptor-binding-site antibodies induces conformational change. Correlation with neutralization potency and analysis of receptor-antibody thermodynamic cycles suggested a receptor-binding-site 'conformational masking' mechanism of neutralization escape. To understand how such an escape mechanism would be compatible with virus-receptor interactions, we tested a soluble dodecameric receptor molecule and found that it neutralized primary HIV-1 isolates with great potency, showing that simultaneous binding of viral envelope glycoproteins by multiple receptors creates sufficient avidity to compensate for such masking. Because this solution is available for cell-surface receptors but not for most antibodies, conformational masking enables HIV-1 to maintain receptor binding and simultaneously to resist neutralization.

Lai A.L., Park H., White J.M., and Tamm L.K. (2006) Fusion peptide of influenza hemagglutinin requires a fixed angle boomerang structure for activity. *J Biol Chem.* **281**, 5760-70.

Abstract: The fusion peptide of influenza hemagglutinin is crucial for cell entry of this virus. Previous studies showed that this peptide adopts a boomerang-shaped structure in lipid model membranes at the pH of membrane fusion. To examine the role of the boomerang in fusion, we changed several residues proposed to stabilize the kink in this structure and measured fusion. Among these, mutants E11A and W14A expressed hemagglutinins with hemifusion and no fusion activities, and F9A and N12A had no effect on fusion, respectively. Binding enthalpies and free energies of mutant peptides to model membranes and their ability to perturb lipid bilayer structures correlated well with the fusion activities of the parent full-length molecules. The structure of W14A determined by NMR and site-directed spin labeling features a flexible kink that points out of the membrane, in sharp contrast to the more ordered boomerang of the wild-type, which points into the membrane. A specific fixed angle boomerang structure is thus required to support membrane fusion.

Langlois C., Mas C., Di L. P., Jenkins L. M., Legault P. and Omichinski J. G. (2008) NMR structure of the complex between the Tfb1 subunit of TFIIF and the activation domain of VP16: structural similarities between VP16 and p53. *J Am. Chem Soc.* **130**, 10596-10604.

Abstract: The Herpes Simplex Virion Protein 16 (VP16) activates transcription through a series of protein/protein interactions involving its highly acidic transactivation domain (TAD). The acidic TAD of VP16 (VP16TAD) has been shown to interact with several partner proteins both in vitro and in vivo, and many of these VP16 partners also bind the acidic TAD of the mammalian tumor suppressor protein p53. For example, the TADs of VP16 and p53 (p53TAD) both interact directly with the p62/Tfb1 (human/yeast) subunit of TFIID, and this interaction correlates with their ability to activate both the initiation and elongation phase of transcription. In this manuscript, we use NMR spectroscopy, isothermal titration calorimetry (ITC) and site-directed mutagenesis studies to characterize the interaction between the VP16TAD and Tfb1. We identify a region within the carboxyl-terminal subdomain of the VP16TAD (VP16C) that has sequence similarity with p53TAD2 and binds Tfb1 with nanomolar affinity. We determine an NMR structure of a Tfb1/VP16C complex, which represents the first high-resolution structure of the VP16TAD in complex with a target protein. The structure demonstrates that like p53TAD2, VP16C forms a 9-residue alpha-helix in complex with Tfb1. Comparison of the VP16/Tfb1 and p53/Tfb1 structures clearly demonstrates how the viral activator VP16C and p53TAD2 shares numerous aspects of binding to Tfb1. Despite the similarities, important differences are observed between the p53TAD2/Tfb1 and VP16C/Tfb1 complexes, and these differences demonstrate how selected activators such as p53 depend on phosphorylation events to selectively regulate transcription

Lario P. I., Pfuetzner R. A., Frey E. A., Creagh L., Haynes C., Maurelli A. T., and Strynadka N. C. (2005) Structure and biochemical analysis of a secretin pilot protein. *EMBO J* **24**, 1111-1121.

Abstract: The ability to translocate virulence proteins into host cells through a type III secretion apparatus (TTSS) is a hallmark of several Gram-negative pathogens including Shigella, Salmonella, Yersinia, Pseudomonas, and enteropathogenic Escherichia coli. In common with other types of bacterial secretion apparatus, the assembly of the TTSS complex requires the preceding formation of its integral outer membrane secretin ring component. We have determined at 1.5 Å the structure of MxiM28-142, the Shigella pilot protein that is essential for the assembly and membrane association of the Shigella secretin, MxiD. This represents the first atomic structure of a secretin pilot protein from the several bacterial secretion systems containing an orthologous secretin component. A deep hydrophobic cavity is observed in the novel 'cracked barrel' structure of MxiM, providing a specific binding domain for the acyl chains of bacterial lipids, a proposal that is supported by our various lipid/MxiM complex structures. Isothermal titration analysis shows that the C-terminal domain of the secretin, MxiD525-570, hinders lipid binding to MxiM.

Leavitt S. A., SchOn A., Klein J. C., Manjappara U., Chaiken I. M., and Freire E. (2004) Interactions of HIV-1 proteins gp120 and Nef with cellular partners define a novel allosteric paradigm. *Curr Protein Pept Sci* **5**, 1-8.

Abstract: During the course of infection, a subset of HIV-1 proteins interacts with multiple cellular partners, sometimes in a hierarchical or sequential way. These proteins include those associated with the initial infection event, with the preparation of the cell for the replicative cycle of the virus and with the exit of new virions from the infected cell. It appears that the interactions of viral proteins with multiple cellular partners are mediated by the occurrence of ligand-induced conformational changes that direct the binding of these proteins to subsequent partners. Two of the most studied HIV-1 proteins that are known to interact with different cellular partners are gp120 and Nef. Here we discuss the interactions of these two proteins with their cellular partners and present new results indicating that the conformational changes undergone by these proteins define a novel allosteric paradigm. In the traditional view, conformational changes are thought to occur between well defined structural conformations of a protein. In gp120 and Nef, those changes involve conformations characterized by the presence of large regions devoid of stable secondary or tertiary structure. Those unstructured regions contain the binding determinants for subsequent partners and only become functionally competent by ligand-induced structuring or un-structuring of those regions. By switching binding epitopes between structured and unstructured conformations the binding affinity can be modulated by several orders of magnitude, thus effectively precluding binding against unwanted partners. A better understanding of these interactions would lead to improved strategies for inhibitor design against these viral targets.

Li T. K., Barbieri C. M., Lin H. C., Rabson A. B., Yang G., Fan Y., Gaffney B. L., Jones R. A., and Pilch D. S. (2004) Drug targeting of HIV-1 RNA/DNA hybrid structures: thermodynamics of recognition and

impact on reverse transcriptase-mediated ribonuclease H activity and viral replication. *Biochemistry* **43**, 9732-9742.

Abstract: RNA degradation via the ribonuclease H (RNase H) activity of human immunodeficiency virus type I (HIV-1) reverse transcriptase (RT) is a critical component of the reverse transcription process. In this connection, mutations of RT that inactivate RNase H activity result in noninfectious virus particles. Thus, interfering with the RNase H activity of RT represents a potential vehicle for the inhibition of HIV-1 replication. Here, we demonstrate an approach for inhibiting the RNase H activity of HIV-1 RT by targeting its RNA-DNA hybrid substrates. Specifically, we show that the binding of the 4,5-disubstituted 2-deoxystreptamine aminoglycosides, neomycin, paromomycin, and ribostamycin, to two different chimeric RNA-DNA duplexes, which mimic two distinct intermediates in the reverse transcription process, inhibits specific RT-mediated RNase H cleavage, with this inhibition being competitive in nature. UV melting and isothermal titration calorimetry studies reveal a correlation between the relative binding affinities of the three drugs for each of the chimeric RNA-DNA host duplexes and the relative extents to which the drugs inhibit RT-mediated RNase H cleavage of the duplexes. Significantly, this correlation also extends to the relative efficacies with which the drugs inhibit HIV-1 replication. In the aggregate, our results highlight a potential strategy for AIDS chemotherapy that should not be compromised by the unusual genetic diversity of HIV-1.

Li Y., Han X., and Tamm L. K. (2003) Thermodynamics of fusion peptide-membrane interactions. *Biochemistry* **42**, 7245-7251.

Abstract: The fusion peptides of viral membrane fusion proteins play a key role in the mechanism of viral spike glycoprotein mediated membrane fusion. These peptides insert into the lipid bilayers of cellular target membranes where they adopt mostly helical secondary structures. To better understand how membranes may be converted to high-energy intermediates during fusion, it is of interest to know how much energy, enthalpy and entropy, is provided by the insertion of fusion peptides into lipid bilayers. Here, we describe a detailed thermodynamic analysis of the binding of analogues of the influenza hemagglutinin fusion peptide of different lengths and amino acid compositions. In small unilamellar vesicles, the interaction of these peptides with lipid bilayers is driven by enthalpy (-16.5 kcal/mol) and opposed by entropy (-30 cal mol⁻¹ K⁻¹). Most of the driving force ($\Delta G = -7.6$ kcal/mol) comes from the enthalpy of peptide insertion deep into the lipid bilayer. Enthalpic gains and entropic losses of peptide folding in the lipid bilayer cancel to a large extent and account for only about 40% of the total binding free energy. The major folding event occurs in the N-terminal segment of the fusion peptide. The C-terminal segment mainly serves to drive the N-terminus deep into the membrane. The fusion-defective mutations G1S, which causes hemifusion, and particularly G1V, which blocks fusion, have major structural and thermodynamic consequences on the insertion of fusion peptides into lipid bilayers. The magnitudes of the enthalpies and entropies of binding of these mutant peptides are reduced, their helix contents are reduced, but their energies of self-association at the membrane surface are increased compared to the wild-type fusion peptide.

Li Y., Han X., Lai A.L., Bushweller J.H., Cafiso D.S., and Tamm L.K.. (2005) Membrane structures of the hemifusion-inducing fusion peptide mutant G1S and the fusion-blocking mutant G1V of influenza virus hemagglutinin suggest a mechanism for pore opening in membrane fusion. *J Virol.* **79**, 12065-76.

Abstract: Influenza virus hemagglutinin (HA)-mediated membrane fusion is initiated by a conformational change that releases a V-shaped hydrophobic fusion domain, the fusion peptide, into the lipid bilayer of the target membrane. The most N-terminal residue of this domain, a glycine, is highly conserved and is particularly critical for HA function; G1S and G1V mutant HAs cause hemifusion and abolish fusion, respectively. We have determined the atomic resolution structures of the G1S and G1V mutant fusion domains in membrane environments. G1S forms a V with a disrupted "glycine edge" on its N-terminal arm and G1V adopts a slightly tilted linear helical structure in membranes. Abolishment of the kink in G1V results in reduced hydrophobic penetration of the lipid bilayer and an increased propensity to form beta-structures at the membrane surface. These results underline the functional importance of the kink in the fusion peptide and suggest a structural role for the N-terminal glycine ridge in viral membrane fusion.

Liu X., Clements A., Zhao K., and Marmorstein R. (2006) Structure of the human Papillomavirus E7 oncoprotein and its mechanism for inactivation of the retinoblastoma tumor suppressor. *J Biol Chem.* **281**, 578-86.

Abstract: The E7 oncoprotein from human Papillomavirus (HPV) mediates cell transformation in part by binding to the human pRb tumor suppressor protein and E2F transcription factors, resulting in the dissociation of pRb from E2F transcription factors and the premature cell progression into the S-phase of the cell cycle. This activity is mediated by the LXCXE motif and the CR3 zinc binding domain of the E7 protein. In this study we report the x-ray crystal structure of the CR3 region of HPV E7 and a structure-based mutational analysis to investigate its mode of pRb and E2F binding and E2F displacement from pRb. The structure reveals a novel zinc-bound E7-CR3 obligate homodimer that contains two surface patches of sequence conservation. Mutation of residues within these patches reveals that one patch is required for pRb binding, whereas the other is required for E2F binding. We also show that both E7-mediated interactions are required to disrupt pRb.E2F complexes. Based on these studies we present a mechanistic model for how E7 displaces E2F from pRb. Because the CR3 region of HPV E7 has no detectable homology to other human proteins, the structure-function studies presented here provide an avenue for developing small molecule compounds that inhibit HPV-E7-mediated cell transformation.

Liu Y., Henry G. D., Hegde R. S. and Baleja J. D. (2007) Solution structure of the hDlg/SAP97 PDZ2 domain and its mechanism of interaction with HPV-18 papillomavirus E6 protein. *Biochemistry* **46**, 10864-10874.

Abstract: The E6 protein from high-risk types of human papillomavirus (HPV) binds PDZ-domain containing proteins and targets them for degradation. We used isothermal titration calorimetry to measure the interaction of a peptide from the C-terminus of HPV-18 E6 to the second PDZ domain (PDZ2) from the human homologue of the Drosophila discs large tumor suppressor protein (hDlg). Isothermal titration calorimetry experiments with a series of peptides showed that HPV-18 E6 bound hDlg PDZ2 about 5-fold stronger than HPV-16 E6, that the contribution of Arg154 to binding was about 1 kcal/mol, and that the binding was disabled by phosphorylation at Thr156. We then used NMR to determine the solution structure of the complex of PDZ2 bound to the HPV-18 E6 peptide. The resultant structures were of high quality and had backbone root-mean-square deviations of less than 0.5 Å. The structure shows a novel mode of interaction in which six residues of the HPV-18 E6 peptide are contacted by the PDZ2 domain, in contrast to the typical four residues used by class I PDZ domains. Molecular dynamics simulations supported a model in which the C- and N-terminal ends of the peptide had different mobilities within the complex. Comparison of the NMR complex structure to previously determined X-ray structures of PDZ2 by itself and bound to different peptides allows a description of conformational changes required for PDZ2 to bind to HPV-18 E6.

Loregian A., Appleton B. A., Hogle J. M., and Coen D. M. (2004) Specific residues in the connector loop of the human cytomegalovirus DNA polymerase accessory protein UL44 are crucial for interaction with the UL54 catalytic subunit. *J Virol* **78**, 9084-9092.

Abstract: The human cytomegalovirus DNA polymerase includes an accessory protein, UL44, which has been proposed to act as a processivity factor for the catalytic subunit, UL54. How UL44 interacts with UL54 has not yet been elucidated. The crystal structure of UL44 revealed the presence of a connector loop analogous to that of the processivity subunit of herpes simplex virus DNA polymerase, UL42, which is crucial for interaction with its cognate catalytic subunit, UL30. To investigate the role of the UL44 connector loop, we replaced each of its amino acids (amino acids 129 to 140) with alanine. We then tested the effect of each substitution on the UL44-UL54 interaction by glutathione S-transferase pulldown and isothermal titration calorimetry assays, on the stimulation of UL54-mediated long-chain DNA synthesis by UL44, and on the binding of UL44 to DNA-cellulose columns. Substitutions that affected residues 133 to 136 of the connector loop measurably impaired the UL44-UL54 interaction without altering the ability of UL44 to bind DNA. One substitution, I135A, completely disrupted the binding of UL44 to UL54 and inhibited the ability of UL44 to stimulate long-chain DNA synthesis by UL54. Thus, similar to the herpes simplex virus UL30-UL42 interaction, a residue of the connector loop of the accessory subunit is crucial for UL54-UL44 interaction. However, while alteration of a polar residue of the UL42 connector loop only partially reduced binding to UL30, substitution of a hydrophobic residue of UL44 completely disrupted the UL54-UL44 interaction. This information may aid the discovery of small-molecule inhibitors of the UL44-UL54 interaction.

Loregian A., Appleton B. A., Hogle J. M., and Coen D. M. (2004) Residues of Human Cytomegalovirus DNA Polymerase Catalytic Subunit UL54 That Are Necessary and Sufficient for Interaction with the Accessory Protein UL44. *J Virol* **78**, 158-167.

Abstract: The human cytomegalovirus DNA polymerase contains a catalytic subunit, UL54, and an accessory protein, UL44. Recent studies suggested that UL54 might interact via its extreme C terminus with UL44 (A. Loregian, R. Rigatti, M. Murphy, E. Schievano, G. Palu', and H. S. Marsden, *J. Virol.* 77:8336-8344, 2003). To address this hypothesis, we quantitatively measured the binding of peptides corresponding to the extreme C terminus of UL54 to UL44 by using isothermal titration calorimetry. A peptide corresponding to the last 22 residues of UL54 was sufficient to bind specifically to UL44 in a 1:1 complex with a dissociation constant of ca. 0.7 μ M. To define individual residues in this segment that are crucial for interacting with UL44, we engineered a series of mutations in the C-terminal region of UL54. The UL54 mutants were tested for their ability to interact with UL44 by glutathione S-transferase pulldown assays, for basal DNA polymerase activity, and for long-chain DNA synthesis in the presence of UL44. We observed that deletion of the C-terminal segment or substitution of alanine for Leu1227 or Phe1231 in UL54 greatly impaired both the UL54-UL44 interaction in pulldown assays and long-chain DNA synthesis without affecting basal polymerase activity, identifying these residues as important for subunit interaction. Thus, like the herpes simplex virus UL30-UL42 interaction, a few specific side chains in the C terminus of UL54 are crucial for UL54-UL44 interaction. However, the UL54 residues important for interaction with UL44 are hydrophobic and not basic. This information might aid in the rational design of new drugs for the treatment of human cytomegalovirus infection.

Lu S., Ge G., and Qi Y. (2004) Ha-VP39 binding to actin and the influence of F-actin on assembly of progeny virions. *Arch Virol* **149**, 2187-2198.

Abstract: We present evidence that actin is necessary for the successful assembly of HaNPV virions. Purified nucleocapsid protein Ha-VP39 of *Heliothis armigera* nuclear polyhedrosis virus (HaNPV) was found to be able to bind to actin in vitro without assistance, as demonstrated by Western blot and isothermal titration calorimeter. ΔH and binding constants (K) detected by isothermal titration calorimeter strongly suggested that Ha-VP39 first binds actin to seed the formation of hexamer complex of actin, and the hexamers then link to each other to form filaments, and the filaments finally twist into cable structures. The proliferation of HaNPV was completely inhibited in Hz-AM1 cells cultivated in the medium containing 0.5 μ g/ml cytochalasin D (CD) to prevent polymerization of actin, while its yield was reduced to 10^{-4} in the presence of 0.1 μ g/ml CD. Actin concentration and the viral DNA synthesis were not significantly affected by CD even though the progeny virions assembled in the CD treated cells were morphologically different from normal ones and resulted in fewer plaques in plaque assay.

Luo Y., Bertero M. G., Frey E. A., Pfuetzner R. A., Wenk M. R., Creagh L., Marcus S. L., Lim D., Sicheri F., Kay C., Haynes C., Finlay B. B., and Strynadka N. C. (2001) Structural and biochemical characterization of the type III secretion chaperones CesT and SigE. *Nat Struct Biol* **8**, 1031-1036.

Abstract: Several Gram-negative bacterial pathogens have evolved a type III secretion system to deliver virulence effector proteins directly into eukaryotic cells, a process essential for disease. This specialized secretion process requires customized chaperones specific for particular effector proteins. The crystal structures of the enterohemorrhagic *Escherichia coli* O157:H7 Tir-specific chaperone CesT and the *Salmonella enterica* SigD-specific chaperone SigE reveal a common overall fold and formation of homodimers. Site-directed mutagenesis suggests that variable, delocalized hydrophobic surfaces observed on the chaperone homodimers are responsible for specific binding to a particular effector protein. Isothermal titration calorimetry studies of Tir-CesT and enzymatic activity profiles of SigD-SigE indicate that the effector proteins are not globally unfolded in the presence of their cognate chaperones.

McKenna S. A., Kim I., Liu C. W., and Puglisi J. D. (2006) Uncoupling of RNA binding and PKR kinase activation by viral inhibitor RNAs. *J Mol Biol* **358**, 1270-1285.

Abstract: Protein kinase RNA-activated (PKR) is a serine/threonine kinase that contains an N-terminal RNA-binding domain and a C-terminal kinase domain. Upon binding double-stranded RNA (dsRNA), PKR can become activated and phosphorylate cellular targets, such as eukaryotic translation initiation factor 2 α (eIF-2 α). Phosphorylation of eIF-2 α results in attenuation of protein translation by the ribosome in either a general or an mRNA-specific manner. Therefore, the interaction between PKR and dsRNAs represents a crucial host cell defense mechanism against viral infection. Viruses can circumvent

PKR function by transcription of virus-encoded dsRNA inhibitors that bind to and inactivate PKR. We present here a biophysical characterization of the interactions between human PKR and two viral inhibitor RNAs, EBER(I) (from Epstein-Barr virus) and VA(I) (from human adenovirus). Autophosphorylation assays confirmed that both EBER(I) and VA(I) are inhibitors of PKR activation, and profiled the kinetics of the inhibition. Binding affinities of dsRNAs to PKR double-stranded RNA-binding domains (dsRBDs) were determined by isothermal titration calorimetry and gel electrophoresis. A single stem-loop domain from each inhibitory RNA mediates the interaction with both dsRBDs of PKR. The binding sites on inhibitor RNAs and the dsRBDs of PKR have been mapped by NMR chemical shift perturbation experiments, which indicate that inhibitors of PKR employ similar surfaces of interaction as activators. Finally, we show that dsRNA binding and inactivation are non-equivalent; regions other than the dsRBD stem-loops of inhibitory RNA are required for inhibition.

McKenna S. A., Lindhout D. A., Shimoike T., Aitken C. E. and Puglisi J. D. (2007) Viral dsRNA inhibitors prevent self-association and autophosphorylation of PKR. *J Mol Biol* **372**, 103-113.

Abstract: Host response to viral RNA genomes and replication products represents an effective strategy to combat viral invasion. PKR is a Ser/Thr protein kinase that binds to double-stranded (ds)RNA, autophosphorylates its kinase domain, and subsequently phosphorylates eukaryotic initiation factor 2 α (eIF2 α). This results in attenuation of protein translation, preventing synthesis of necessary viral proteins. In certain DNA viruses, PKR function can be evaded by transcription of highly structured virus-encoded dsRNA inhibitors that bind to and inactivate PKR. We probe here the mechanism of PKR inhibition by two viral inhibitor RNAs, EBER(I) (from Epstein-Barr) and VA(I) (from human adenovirus). Native gel shift mobility assays and isothermal titration calorimetry experiments confirmed that the RNA-binding domains of PKR are sufficient and necessary for the interaction with dsRNA inhibitors. Both EBER(I) and VA(I) are effective inhibitors of PKR activation by preventing trans-autophosphorylation between two PKR molecules. The RNA inhibitors prevent self-association of PKR molecules, providing a mechanistic basis for kinase inhibition. A variety of approaches indicated that dsRNA inhibitors remain associated with PKR under activating conditions, as opposed to activator dsRNA molecules that dissociate due to reduced affinity for the phosphorylated form of PKR. Finally, we show using a HeLa cell extract system that inhibitors of PKR result in translational recovery by the protein synthesis machinery. These data indicate that inhibitory dsRNAs bind preferentially to the latent, dephosphorylated form of PKR and prevent dimerization that is required for trans-autophosphorylation.

Meher A. K., Bal N. C., Chary K. V., and Arora A. (2006) Mycobacterium tuberculosis H37Rv ESAT-6-CFP-10 complex formation confers thermodynamic and biochemical stability. *FEBS J* **273**, 1445-1462.

Abstract: The 6-kDa early secretory antigenic target (ESAT-6) and culture filtrate protein-10 (CFP-10), expressed from the region of deletion-1 (RD1) of Mycobacterium tuberculosis H37Rv, are known to play a key role in virulence. In this study, we explored the thermodynamic and biochemical changes associated with the formation of the 1 : 1 heterodimeric complex between ESAT-6 and CFP-10. Using isothermal titration calorimetry (ITC), we precisely determined the association constant and free energy change for formation of the complex to be 2×10^7 M⁻¹ and -9.95 kcal.mol⁻¹, respectively. Strikingly, the thermal unfolding of the ESAT-6-CFP-10 heterodimeric complex was completely reversible, with a T(m) of 53.4 degrees C and ΔH of 69 kcal.mol⁻¹. Mixing of ESAT-6 and CFP-10 at any temperature below the T(m) of the complex led to induction of helical conformation, suggesting molecular recognition between specific segments of unfolded ESAT-6 and CFP-10. Enhanced biochemical stability of the complex was indicated by protection of ESAT-6 and an N-terminal fragment of CFP-10 from proteolysis with trypsin. However, the flexible C-terminal of CFP-10 in the complex, which has been shown to be responsible for binding to macrophages and monocytes, was cleaved by trypsin. In the presence of phospholipid membranes, ESAT-6, but not CFP-10 and the complex, showed an increase in alpha-helical content and enhanced thermal stability. Overall, complex formation resulted in structural changes, enhanced thermodynamic and biochemical stability, and loss of binding to phospholipid membranes. These features of complex formation probably determine the physiological role of ESAT-6, CFP-10 and/or the complex in vivo. The ITC and thermal unfolding approach described in this study can readily be applied to characterization of the 11 other pairs of ESAT-6 family proteins and for screening ESAT-6 and CFP-10 mutants.

Mishra S. H., Shelley C. M., Barrow D. J., Jr., Darby M. K., and Germann M. W. (2006) Solution structures and characterization of human immunodeficiency virus Rev responsive element IIB RNA targeting zinc finger proteins. *Biopolymers* **83**, 352-364.

Abstract: The Rev responsive element (RRE), a part of unspliced human immunodeficiency virus (HIV) RNA, serves a crucial role in the production of infectious HIV virions. The viral protein Rev binds to RRE and facilitates transport of mRNA to the cytoplasm. Inhibition of the Rev-RRE interaction disrupts the viral life cycle. Using a phage display protocol, dual zinc finger proteins (ZNFs) were generated that bind specifically to RREIIB at the high affinity Rev binding site. These proteins were further shortened and simplified, and they still retained their RNA binding affinity. The solution structures of ZNF29 and a mutant, ZNF29G29R, have been determined by nuclear magnetic resonance (NMR) spectroscopy. Both proteins form C(2)H(2)-type zinc fingers with essentially identical structures. RNA protein interactions were evaluated quantitatively by isothermal titration calorimetry, which revealed dissociation constants (K_d's) in the nanomolar range. The interaction with the RNA is dependent upon the zinc finger structure; in the presence of EDTA, RNA binding is abolished. For both proteins, RNA binding is mediated by the alpha-helical portion of the zinc fingers and target the bulge region of RREIIB-TR. However, ZNF29G29R exhibits significantly stronger binding to the RNA target than ZNF29; this illustrates that the binding of the zinc finger scaffold is amenable to further improvements.

Munshi U. M., Kim J., Nagashima K., Hurley J. H. and Freed E. O. (2007) An Alix fragment potently inhibits HIV-1 budding: characterization of binding to retroviral YPXL late domains. *J Biol Chem* **282**, 3847-3855.

Abstract: The retroviral structural protein, Gag, contains small peptide motifs known as late domains that promote efficient virus release from the infected cell. In addition to the well characterized PTAP late domain, the p6 region of HIV-1 Gag contains a binding site for the host cell protein Alix. To better understand the functional role of the Gag/Alix interaction, we overexpressed an Alix fragment composed of residues 364-716 (Alix 364-716) and examined the effect on release of wild type (WT) and Alix binding site mutant HIV-1. We observed that Alix 364-716 expression significantly inhibited WT virus release and Gag processing and that mutation of the Alix binding site largely relieved this inhibition. Furthermore, Alix 364-716 expression induced a severe defect on WT but not mutant particle morphology. Intriguingly, the impact of Alix 364-716 expression on HIV-1 release and Gag processing was markedly different from that induced by mutation of the Alix binding site in p6. The association of Alix 364-716 with HIV-1 and equine infectious anemia virus late domains was quantitatively evaluated by isothermal titration calorimetry and surface plasmon resonance techniques, and the effects of mutations in these viral sequences on Alix 364-716 binding was determined. This study identifies a novel Alix-derived dominant negative inhibitor of HIV-1 release and Gag processing and provides quantitative information on the interaction between Alix and viral late domains.

Neu U., Woellner K., Gauglitz G. and Stehle T. (2008) Structural basis of GM1 ganglioside recognition by simian virus 40. *Proc. Natl. Acad. Sci U. S. A* **105**, 5219-5224.

Abstract: Simian virus 40 (SV40) has been a paradigm for understanding attachment and entry of nonenveloped viruses, viral DNA replication, and virus assembly, as well as for endocytosis pathways associated with caveolin and cholesterol. We find by glycan array screening that SV40 recognizes its ganglioside receptor GM1 with a quite narrow specificity, but isothermal titration calorimetry shows that individual binding sites have a relatively low affinity, with a millimolar dissociation constant. The high-resolution crystal structure of recombinantly produced SV40 capsid protein, VP1, in complex with the carbohydrate portion of GM1, reveals that the receptor is bound in a shallow solvent-exposed groove at the outer surface of the capsid. Through a complex network of interactions, VP1 recognizes a conformation of GM1 that is the dominant one in solution. Analysis of contacts provides a structural basis for the observed specificity and suggests binding mechanisms for additional physiologically relevant GM1 variants. Comparison with murine Polyomavirus (Polyoma) receptor complexes reveals that SV40 uses a different mechanism of sialic acid binding, which has implications for receptor binding of human polyomaviruses. The SV40-GM1 complex reveals a parallel to cholera toxin, which uses a similar cell entry pathway and binds GM1 in the same conformation.

Nezami A., Kimura T., Hidaka K., Kiso A., Liu J., Kiso Y., Goldberg D. E., and Freire E. (2003) High-affinity inhibition of a family of Plasmodium falciparum proteases by a designed adaptive inhibitor.

Biochemistry **42**, 8459-8464.

Abstract: Drug development against viral or microbial targets is often compounded by the existence of naturally occurring polymorphisms or drug resistant mutations. In the case of *Plasmodium falciparum*, the etiological agent of malaria, four related and essential proteases, plasmepsin I, II, and IV and the histone-aspartyl protease (HAP), have been identified in the food vacuole of the parasite. Since all of these enzymes are involved in the hemoglobin degradation of infected victims, the simultaneous inhibition of the four enzymes can be expected to lead to a faster starvation of the parasite and to delay the onset of drug resistance, since four enzymes will need to mutate in a concerted fashion. This study describes the design of an adaptive inhibitor intended to inhibit the entire plasmepsin family. Adaptive inhibitors bind with extremely high affinity to a primary target within the family and maintain significant affinity against the remaining members. This objective is accomplished by engineering the strongest and most specific interactions of the inhibitor against conserved regions of the binding site and by accommodating target variations by means of flexible asymmetric functional groups. Using this approach, we have designed an inhibitor with subnanomolar affinity (0.5 nM) against the primary target, plasmepsin II, and with no loss or a very small loss of affinity against plasmepsin IV, I, and HAP ($K(i)$ ratios of 0.4, 7.1, and 17.7, respectively). The core of the inhibitor is defined by an allophenylalanine scaffold. Adaptability is provided by an asymmetric amino indanol functional group facing one of the key variable regions in the binding site. Adaptive inhibitors, which display high affinity against several variations of a primary target, are expected to play an important role in the chemotherapy of infectious diseases.

Nunn C. M., Jeeves M., Cliff M. J., Urquhart G. T., George R. R., Chao L. H., Tsuchia Y., and Djordjevic S. (2005) Crystal Structure of Tobacco Etch Virus Protease Shows the Protein C Terminus Bound within the Active Site. *J Mol Biol* **350**, 145-155.

Abstract: Tobacco etch virus (TEV) protease is a cysteine protease exhibiting stringent sequence specificity. The enzyme is widely used in biotechnology for the removal of the affinity tags from recombinant fusion proteins. Crystal structures of two TEV protease mutants as complexes with a substrate and a product peptide provided the first insight into the mechanism of substrate specificity of this enzyme. We now report a 2.7 Å crystal structure of a full-length inactive C151A mutant protein crystallised in the absence of peptide. The structure reveals the C terminus of the protease bound to the active site. In addition, we determined dissociation constants of TEV protease substrate and product peptides using isothermal titration calorimetry for various forms of this enzyme. Data suggest that TEV protease could be inhibited by the peptide product of autolysis. Separate modes of recognition for native substrates and the site of TEV protease self-cleavage are proposed.

Oddo C., Freire E., Frappier L., and Prat-Gay G. (2006) Mechanism of DNA recognition at a viral replication origin. *J Biol Chem* **281**, 26893-26903.

Abstract: Recognition of the DNA origin by the Epstein-Barr nuclear antigen 1 (EBNA1) protein is the primary event in latent phase genome replication of the Epstein-Barr virus, a model for replication initiation in eukaryotes. We carried out an extensive thermodynamic and kinetic characterization of the binding mechanism of the DNA binding domain of EBNA1, EBNA1452-641, to a DNA fragment containing a single specific origin site. The interaction displays a binding energy of 12.7 kcal mol⁻¹, with 11.9 kcal mol⁻¹ coming from the enthalpic change with a minimal entropic contribution. Formation of the EBNA1452-641.DNA complex is accompanied by a heat capacity change of -1.22 kcal mol⁻¹ K⁻¹, a very large value considering the surface area buried, which we assign to an unusually apolar protein-DNA interface. Kinetic dissociation experiments, including fluorescence anisotropy and a continuous native electrophoretic mobility shift assay, confirmed that two EBNA1.DNA complex conformers are in slow equilibrium; one dissociates slowly ($t_{1/2}$ approximately 41 min) through an undissociated intermediate species and the other corresponds to a fast twostep dissociation route ($t_{1/2}$ approximately 0.8 min). In line with this, at least two parallel association events from two populations of protein conformers are observed, with on-rates of 0.25-1.6x10⁸ m⁻¹ s⁻¹, which occur differentially either in excess protein or DNA molecules. Both parallel complexes undergo subsequent firstorder rearrangements of approximately 2.0 s⁻¹ to yield two consolidated complexes. These parallel association and dissociation routes likely allow additional flexible regulatory events for site recognition depending on site availability according to nucleus environmental conditions, which may lock a final recognition event, dissociate and re-bind, or slide along the DNA.

Ohtaka H., Schon A., and Freire E. (2003) Multidrug resistance to HIV-1 protease inhibition requires cooperative coupling between distal mutations. *Biochemistry* **42**, 13659-13666.

Abstract: The appearance of viral strains that are resistant to protease inhibitors is one of the most serious problems in the chemotherapy of HIV-1/AIDS. The most pervasive drug-resistant mutants are those that affect all inhibitors in clinical use. In this paper, we have characterized a multiple-drug-resistant mutant of the HIV-1 protease that affects indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir. This mutant (MDR-HM) contains six amino acid mutations (L101/M46I/I54V/V82A/I84V/L90M) located within and outside the active site of the enzyme. Microcalorimetric and enzyme kinetic measurements indicate that this mutant lowers the affinity of all inhibitors by 2-3 orders of magnitude. By comparison, the multiple-drug-resistant mutant only increased the K_m of the substrate by a factor of 2, indicating that the substrate is able to adapt to the changes caused by the mutations and maintain its binding affinity. To understand the origin of resistance, three submutants containing mutations in specific regions were also studied, i.e., the active site (V82A/I84V), flap region (M46I/I54V), and dimerization region (L101/L90M). None of these sets of mutations by themselves lowered the affinity of inhibitors by more than 1 order of magnitude, and additionally, the sum of the effects of each set of mutations did not add up to the overall effect, indicating the presence of cooperative effects. A mutant containing only the four active site mutations (V82A/I84V/M46I/I54V) only showed a small cooperative effect, suggesting that the mutations at the dimer interface (L101/L90M) play a major role in eliciting a cooperative response. These studies demonstrate that cooperative interactions contribute an average of 1.2 ± 0.7 kcal/mol to the overall resistance, most of the cooperative effect (0.8 ± 0.7 kcal/mol) being mediated by the mutations at the dimerization interface. Not all inhibitors in clinical use are affected the same by long-range cooperative interactions between mutations. These interactions can amplify the effects of individual mutations by factors ranging between 2 and 40 depending on the inhibitor. Dissection of the energetics of drug resistance into enthalpic and entropic components provides a quantitative account of the inhibitor response and a set of thermodynamic guidelines for the design of inhibitors with a lower susceptibility to this type of mutations.

O'Keefe B. R., Shenoy S. R., Xie D., Zhang W., Muschik J. M., Currens M. J., Chaiken I., and Boyd M. R. (2000) Analysis of the interaction between the HIV-inactivating protein cyanovirin-N and soluble forms of the envelope glycoproteins gp120 and gp41. *Mol Pharmacol* **58**, 982-992.

Abstract: The novel virucidal protein cyanovirin-N (CV-N) binds with equally high affinity to soluble forms of either H9 cell-produced or recombinant glycosylated HIV-1 gp120 (sgp120) or gp160 (sgp160). Fluorescence polarization studies showed that CV-N is also capable of binding to the glycosylated ectodomain of the HIV-envelope protein gp41 (sgp41) (as well as SIV glycoprotein 32), albeit with considerably lower affinity than the sgp120/CV-N interaction. Pretreatment of CV-N with either sgp120 or sgp41 abrogated the neutralizing activity of CV-N against intact, infectious HIV-1 virions. Isothermal calorimetry and optical biosensor binding studies showed that CV-N bound to recombinant sgp120 with a K_d value ranging from 2 to 45 nM and to sgp41 with a K_d value of 606 nM; furthermore, they indicated an approximate 5:1 stoichiometry for CV-N binding to sgp120 and a 1:1 stoichiometry for CV-N binding to sgp41. Circular dichroism studies additionally illuminated the binding of CV-N with both sgp120 and sgp41, providing the first direct evidence that conformational changes are a consequence of CV-N interactions with both HIV-1 envelope glycoproteins.

Pancera M., Lebowitz J., Schon A., Zhu P., Freire E., Kwong P. D., Roux K. H., Sodroski J., and Wyatt R. (2005) Soluble mimetics of human immunodeficiency virus type 1 viral spikes produced by replacement of the native trimerization domain with a heterologous trimerization motif: characterization and ligand binding analysis. *J Virol* **79**, 9954-9969.

Abstract: The human immunodeficiency virus type 1 (HIV-1) exterior envelope glycoprotein, gp120, mediates binding to the viral receptors and, along with the transmembrane glycoprotein gp41, is a major target for neutralizing antibodies. We asked whether replacing the gp41 fusion/trimerization domain with a stable trimerization motif might lead to a more stable gp120 trimer that would be amenable to structural and immunologic analysis. To obtain stable gp120 trimers, a heterologous trimerization motif, GCN4, was appended to the C terminus of YU2gp120. Biochemical analysis indicated that the gp120-GCN4 trimers were superior to gp140 molecules in their initial homogeneity, and trilobed structures were observable by electron microscopy. Biophysical analysis of gp120-GCN4 trimers by isothermal titration calorimetry (ITC) and ultracentrifugation analyses indicated that most likely two molecules of soluble CD4 could bind

to one gp120-GCN4 trimer. To further examine restricted CD4 stoichiometric binding to the gp120-GCN4 trimers, we generated a low-affinity CD4 binding trimer by introducing a D457V change in the CD4 binding site of each gp120 monomeric subunit. The mutant trimers could definitively bind only one soluble CD4 molecule, as determined by ITC and sedimentation equilibrium centrifugation. These data indicate that there are weak interactions between the gp120 monomeric subunits of the GCN4-stabilized trimers that can be detected by low-affinity ligand sensing. By similar analysis, we also determined that removal of the variable loops V1, V2, and V3 in the context of the gp120-GCN4 proteins allowed the binding of three CD4 molecules per trimer. Interestingly, both the gp120-GCN4 variants displayed a restricted stoichiometry for the CD4-induced antibody 17b of one antibody molecule binding per trimer. This restriction was not evident upon removal of the variable loops V1 and V2 loops, consistent with conformational constraints in the wild-type gp120 trimers and similar to those inherent in the functional Env spike. Thus, the gp120-GCN4 trimers demonstrate several properties that are consistent with some of those anticipated for gp120 in the context of the viral spike.

Panyukov Y. V., Nemykh M. A., Dobrov E. N. and Drachev V. A. (2007) Surfactant-Induced Amorphous Aggregation of Tobacco Mosaic Virus Coat Protein: A Physical Methods Approach. *Macromol. Biosci* **8**, 199-209.

Abstract: The interactions of non-ionic surfactant Triton X-100 and the coat protein of tobacco mosaic virus, which is an established model for both ordered and non-ordered protein aggregation, were studied using turbidimetry, differential scanning calorimetry, isothermal titration calorimetry, and dynamic light scattering. It was found that at the critical aggregation concentration (equal to critical micelle concentration) of 138×10^{-6} M, Triton X-100 induces partial denaturation of tobacco mosaic virus coat protein molecules followed by protein amorphous aggregation. Protein aggregation has profound ionic strength dependence and proceeds due to hydrophobic sticking of surfactant-protein complexes (start aggregates) with initial radii of 46 nm. It has been suggested that the anionic surfactant sodium dodecyl sulfate forms mixed micelles with Triton X-100 and therefore reverses protein amorphous aggregation with release of protein molecules from the amorphous aggregates. A stoichiometric ratio of 5 was found for Triton X-100-sodium dodecyl sulfate interactions.

Parker M. H., Brouillette C. G., and Prevelige P. E., Jr. (2001) Kinetic and calorimetric evidence for two distinct scaffolding protein binding populations within the bacteriophage P22 procapsid. *Biochemistry* **40**, 8962-8970.

Abstract: A wide variety of viruses require the transient presence of scaffolding proteins to direct capsid assembly. In the case of bacteriophage P22, a model in which the scaffolding protein selectively stabilizes on-pathway growing intermediates has been proposed. The stoichiometry and thermodynamics of binding of the bacteriophage P22 scaffolding protein within the procapsid were analyzed by light scattering and isothermal titration calorimetry. Calorimetric experiments carried out between 10 and 37 degrees C were consistent with the presence of at least two distinct populations of binding sites, in agreement with kinetic evidence obtained by a light scattering assay. Binding to the high-affinity sites occurred at 20 degrees C with a stoichiometry of approximately 60 scaffolding molecules per procapsid and an apparent K_d of approximately 100-300 nM and was almost completely enthalpy-driven. For the second binding population, precise fitting of the data was impossible due to small heats of binding, but the thermodynamics of binding were clearly distinct from the high-affinity phase. The heat capacity change (ΔC_p) of binding was large for the high-affinity sites and negative for both sets of sites. Addition of sodium chloride (1 M) greatly reduced the magnitude of the apparent ΔH , in agreement with previous evidence that electrostatic interactions play a major role in binding. A mutant scaffolding protein that forms covalent dimers (R74C/L177I) bound only to the high-affinity sites. These data comprise the first quantitative measurements of the energetics of the coat protein/scaffolding protein interaction.

Perozzo R., Jelesarov I., Bosshard H. R., Folkers G., and Scapozza L. (2000) Compulsory order of substrate binding to herpes simplex virus type 1 thymidine kinase. A calorimetric study. *J Biol Chem* **275**, 16139-16145.

Abstract: Isothermal titration calorimetry has been used to investigate the thermodynamic parameters of the binding of thymidine (dT) and ATP to herpes simplex virus type 1 thymidine kinase (HSV1 TK). Binding follows a sequential pathway in which dT binds first and ATP second. The free enzyme does not bind ATP, whose binding site becomes only accessible in the HSV1 TK.dT complex. At pH 7.5 and 25

degrees C, the binding constants are $1.9 \times 10^5 \text{ m}^{-1}$ for dT and $3.9 \times 10^6 \text{ m}^{-1}$ for ATP binding to the binary HSV1 TK.dT complex. Binding of both substrates is enthalpy-driven and opposed by a large negative entropy change. The heat capacity change (ΔC_p) obtained from ΔH in the range of 10-25 degrees C is $-360 \text{ cal K}^{-1} \text{ mol}^{-1}$ for dT binding and $-140 \text{ cal K}^{-1} \text{ mol}^{-1}$ for ATP binding. These large ΔC_p values are incompatible with a rigid body binding model in which the dT and ATP binding sites pre-exist in the free enzyme. Values of ΔC_p and $T\Delta S$ strongly indicate large scale conformational adaptation of the active site in sequential substrate binding. The conformational changes seem to be more pronounced in dT binding than in the subsequent ATP binding. Considering the crystal structure of the ternary HSV1 TK.dT.ATP complex, a large movement in the dT binding domain and a smaller but substantial movement in the LID domain are proposed to take place when the enzyme changes from the substrate-free, presumably more open and less ordered conformation to the closed and compact conformation of the ternary enzyme-substrate complex.

Pilger B. D., Perozzo R., Alber F., Wurth C., Folkers G., and Scapozza L. (1999) Substrate diversity of herpes simplex virus thymidine kinase. Impact Of the kinematics of the enzyme. *J Biol Chem* **274**, 31967-31973.

Abstract: Herpes simplex virus type 1 (HSV 1) thymidine kinase (TK) exhibits an extensive substrate diversity for nucleobases and sugar moieties, in contrast to other TKs. This substrate diversity is the crucial molecular basis of selective antiviral and suicide gene therapy. The mechanisms of substrate binding of HSV 1 TK were studied by means of site-directed mutagenesis combined with isothermal calorimetric measurements and guided by theoretical calculations and sequence comparison. The results show the link between the exceptionally broad substrate diversity of HSV 1 TK and the presence of structural features such as the residue triad His-58/Met-128/Tyr-172. The mutation of Met-128 into a Phe and the double mutant M128F/Y172F result in mutants that have lost their activity. However, by exchanging His to form the triple mutant H58L/M128F/Y172F, the enzyme regains activity. Strikingly, this triple mutant becomes resistant toward acyclovir. Furthermore, we give evidence for the importance of Glu-225 of the flexible LID region for the catalytic reaction. The data presented give new insights to understand mechanisms ruling substrate diversity and thus are crucial for both the development of new antiviral drugs and engineering of mutant TKs apt to accept novel substrate analogs for gene therapeutic approaches.

Praefcke G. J., Geyer M., Schwemmler M., Robert K. H., and Herrmann C. (1999) Nucleotide-binding characteristics of human guanylate-binding protein 1 (hGBP1) and identification of the third GTP-binding motif. *J Mol Biol* **292**, 321-332.

Abstract: hGBP1 is a GTPase with antiviral activity encoded by an interferon- activated human gene. Specific binding of hGBP1 to guanine nucleotides has been established although only two classical GTP-binding motifs were found in its primary sequence. The unique position of hGBP1 amongst known GTPases is further demonstrated by the hydrolysis of GTP to GDP and GMP. Although subsequent cleavage of orthophosphates rather than pyrophosphate was demonstrated, GDP coming from bulk solution cannot serve as a substrate. The relation of guanine nucleotide binding and hydrolysis to the antiviral function of hGBP1 is unknown. Here we show similar binding affinities for all three guanine nucleotides and the ability of both products, GDP and GMP, to compete with GTP binding. Fluorimetry and isothermal titration calorimetry were applied to prove that only one nucleotide binding site is present in hGBP1. Furthermore, we identified the third canonical GTP-binding motif and verified its role in nucleotide recognition by mutational analysis. The high guanine nucleotide dissociation rates measured by stopped-flow kinetics are responsible for the weak affinities to hGBP1 when compared to other GTPases like Ras or Galpha. By means of fluorescence and NMR spectroscopy it is demonstrated that aluminium fluoride forms a complex with hGBP1 only in the GDP state, presumably mimicking the transition state of GTP hydrolysis. Tentatively, the involvement of a GAP domain in hGBP1 in GTP hydrolysis is suggested. These results will serve as a basis for the determination of the differential biological functions of the three nucleotide states and for the elucidation of the unique mechanism of nucleotide hydrolysis catalysed by hGBP1.

Randell J.C., Komazin G., Jiang C., Hwang C.B., and Coen DM. (2005) Effects of substitutions of arginine residues on the basic surface of herpes simplex virus UL42 support a role for DNA binding in processive DNA synthesis. *J Virol.* **79**, 12025-34.

Abstract: The way that UL42, the processivity subunit of the herpes simplex virus DNA polymerase, interacts with DNA and promotes processivity remains unclear. A positively charged face of UL42 has been proposed to participate in electrostatic interactions with DNA that would tether the polymerase to a template without preventing its translocation via DNA sliding. An alternative model proposes that DNA binding by UL42 is not important for processivity. To investigate these issues, we substituted alanine for each of four conserved arginine residues on the positively charged surface. Each single substitution decreased the DNA binding affinity of UL42, with 14- to 30-fold increases in apparent dissociation constants. The mutant proteins exhibited no meaningful change in affinity for binding to the C terminus of the catalytic subunit of the polymerase, indicating that the substitutions exert a specific effect on DNA binding. The substitutions decreased UL42-mediated long-chain DNA synthesis by the polymerase in the same rank order in which they affected DNA binding, consistent with a role for DNA binding in polymerase processivity. Combining these substitutions decreased DNA binding further and impaired the complementation of a UL42 null virus in transfected cells. Additionally, using a revised mathematical model to analyze rates of dissociation of UL42 from DNAs of various lengths, we found that dissociation from internal sites, which would be the most important for tethering the polymerase, was relatively slow, even at ionic strengths that permit processive DNA synthesis by the holoenzyme. These data provide evidence that the basic surface of UL42 interacts with DNA and support a model in which DNA binding by UL42 is important for processive DNA synthesis.

Rangachari V., Marin V., Bienkiewicz E. A., Semavina M., Guerrero L., Love J. F., Murphy J. R., and Logan T. M. (2005) Sequence of ligand binding and structure change in the diphtheria toxin repressor upon activation by divalent transition metals. *Biochemistry* **44**, 5672-5682.

Abstract: The diphtheria toxin repressor (DtxR) is an Fe(II)-activated transcriptional regulator of iron homeostatic and virulence genes in *Corynebacterium diphtheriae*. DtxR is a two-domain protein that contains two structurally and functionally distinct metal binding sites. Here, we investigate the molecular steps associated with activation by Ni(II)Cl₂ and Cd(II)Cl₂. Equilibrium binding energetics for Ni(II) were obtained from isothermal titration calorimetry, indicating apparent metal dissociation constants of 0.2 and 1.7 μM for two independent sites. The binding isotherms for Ni(II) and Cd(II) exhibited a characteristic exothermic-endothermic pattern that was used to infer the metal binding sequence by comparing the wild-type isotherm with those of several binding site mutants. These data were complemented by measuring the distance between specific backbone amide nitrogens and the first equivalent of metal through heteronuclear NMR relaxation measurements. Previous studies indicated that metal binding affects a disordered to ordered transition in the metal binding domain. The coupling between metal binding and structure change was investigated using near-UV circular dichroism spectroscopy. Together, the data show that the first equivalent of metal is bound by the primary metal binding site. This binding orients the DNA binding helices and begins to fold the N-terminal domain. Subsequent binding at the ancillary site completes the folding of this domain and formation of the dimer interface. This model is used to explain the behavior of several mutants.

Rozak D. A., Orban J., and Bryan P. N. (2005) G148-GA3: a streptococcal virulence module with atypical thermodynamics of folding optimally binds human serum albumin at physiological temperatures. *Biochim Biophys Acta* **1753**, 226-233.

Abstract: The third albumin binding domain of streptococcal protein G strain 148 (G148-GA3) belongs to a novel class of prokaryotic albumin binding modules that is thought to support virulence in several bacterial species. Here, we characterize G148-GA3 folding and albumin binding by using differential scanning calorimetry and isothermal titration calorimetry to obtain the most complete set of thermodynamic state functions for any member of this medically significant module. When buffered at pH 7.0 the 46-amino acid alpha-helical domain melts at 72 degrees C and exhibits marginal stability (15 kJ/mol) at 37 degrees C. G148-GA3 unfolding is characterized by small contributions to entropy from non-hydrophobic forces and a low ΔC_p (1.1 kJ/(deg mol)). Isothermal titration calorimetry reveals that the domain has evolved to optimally bind human serum albumin near 37 degrees C with a binding constant of 1.4 × 10⁷ M⁻¹. Analysis of G148-GA3 thermodynamics suggests that the domain experiences atypically small per residue changes in structural dynamics and heat capacity while transiting between folded and unfolded states.

Rozak D. A., Alexander P. A., He Y., Chen Y., Orban J., and Bryan P. N. (2006) Using offset recombinant polymerase chain reaction to identify functional determinants in a common family of bacterial albumin binding domains. *Biochemistry* **45**, 3263-3271.

Abstract: The 46 amino acid GA albumin binding module is a putative virulence factor that has been identified in 16 domains from four bacterial species. Aside from their possible effects on pathogenicity and host specificity, the natural genotypic and phenotypic variations that exist among members of this module offer unique opportunities for researchers to identify and explore functional determinants within the well-defined sequence space. We used a recently developed in vitro recombination technique, known as offset recombinant PCR, to shuffle seven homologues that encode a broad range of natural GA polymorphisms. Phage display and selection were applied to probe the recombinant library for members that showed simultaneous improvements to human and guinea pig serum albumin binding. Thermodynamic data for the most common phage-selected mutant suggest that domain-stabilizing mutations substantially improved GA binding for both species of albumin.

Rudiger H., Siebert H. C., Solis D., Jimenez-Barbero J., Romero A., Der Lieth C. W., Diaz-Marino T., and Gabius H. J. (2000) Medicinal chemistry based on the sugar code: fundamentals of lectinology and experimental strategies with lectins as targets. *Curr Med Chem* **7**, 389-416.

Abstract: Theoretical calculations reveal that oligosaccharides are second to no other class of biochemical oligomery in terms of coding capacity. As integral part of cellular glycoconjugates they can serve as recognitive units for receptors (lectins). Having first been detected in plants, lectins are present ubiquitously. Remarkably for this field, they serve as bacterial and viral adhesins. Following a description of these branches of lectinology to illustrate history, current status and potential for medicinal chemistry, we document that lectins are involved in a wide variety of biochemical processes including intra- and intercellular glycoconjugate trafficking, initiation of signal transduction affecting e. g. growth regulation and cell adhesion in animals. It is thus justified to compare crucial carbohydrate epitopes with the postal code ensuring correct mail routing and delivery. In view of the functional relevance of lectins the design of high-affinity reagents to occupy their carbohydrate recognition domains offers the perspective for an attractive source of new drugs. Their applications can be supposed to encompass the use as cell-type-selective determinant for targeted drug delivery and as blocking devices in anti-adhesion therapy during infections and inflammatory disease. To master the task of devising custom-made glycans/glycomimetics for this purpose, the individual enthalpic and entropic contributions in the molecular rendezvous between the sugar receptor under scrutiny and its ligand in the presence of solvent molecules undergoing positional rearrangements need to be understood and rationally exploited. As remunerative means to this end, cleverly orchestrated deployment of a panel of methods is essential. Concerning the carbohydrate ligand, its topological parameters and flexibility are assessed by the combination of computer-assisted molecular-mechanics and molecular-dynamics calculations and NMR-spectroscopic measurements. In the presence of the receptor, the latter technique will provide insights into conformational aspects of the bound ligand and into spatial vicinity of the ligand to distinct side chains of amino acids establishing the binding site in solution. Also in solution, the hydrogen-bonding pattern in the complex can be mapped with monodeoxy and monofluoro derivatives of the oligosaccharide. Together with X-ray crystallographic and microcalorimetric studies the limits of a feasible affinity enhancement can be systematically probed. With galactoside-binding lectins as instructive model, recent progress in this area of drug design will be documented, emphasizing the general applicability of the outlined interdisciplinary approach.

Sarver R. W., Rogers J. M., Stockman B. J., Epps D. E., DeZwaan J., Harris M. S., and Baldwin E. T. (2002) Physical methods to determine the binding mode of putative ligands for hepatitis C virus NS3 helicase. *Anal Biochem* **309**, 186-195.

Abstract: Several small molecules identified by high-throughput screening (HTS) were evaluated for their ability to bind to a nonstructural protein 3 (NS3) helicase from hepatitis C virus (HCV). Equilibrium dissociation constants (K_d's) of the compounds for this helicase were determined using several techniques including an assay measuring the kinetics of isothermal enzyme denaturation at several concentrations of the test molecule. Effects of two nonhydrolyzable ATP analogs on helicase denaturation were measured as controls using the isothermal denaturation (ITD) assay. Two compounds, 4-(2,4-dimethylphenyl)-2,7,8-trimethyl-4,5-quinolinediamine and 2-phenyl-N-(5-piperazin-1-yl)pentylquinazolin-4-amine, were identified from screening that inhibited the enzyme and had low micromolar dissociation constants for NS3 helicase in the ITD assay. Low micromolar affinity of the quinolinediamine to helicase was also confirmed

by nuclear magnetic resonance experiments. Unfortunately, isothermal titration calorimetry (ITC) experiments indicated that a more water-soluble analog bound to the 47/23-mer oligonucleotide helicase substrate with low micromolar affinity as did the substituted quinazolinamine. There was no further interest in these templates as helicase inhibitors due to the nonspecific binding to enzyme and substrate. A combination of physical methods was required to discern the mode of action of compounds identified by HTS and remove undesirable lead templates from further consideration.

Sawada T., Hashimoto T., Nakano H., Suzuki T., Suzuki Y., Kawaoka Y., Ishida H. and Kiso M. (2007) Influenza viral hemagglutinin complicated shape is advantageous to its binding affinity for sialosaccharide receptor. *Biochem Biophys Res Commun* **355**, 6-9.

Abstract: Do the complexity and the bulkiness of a protein affect the affinity between protein and ligand? We attempted to investigate this problem by using ab initio fragment molecular orbital (FMO) method to calculate the binding energy between human influenza viral hemagglutinin (HA) and human oligosaccharide receptor. We compared the binding energies of 4 different sizes of human A virus HA H3 subtype complexed with human receptor Neu5Ac(alpha2-6)Gal as a model. The full shape receptor binding domain complexed with Neu5Ac(alpha2-6)Gal had the highest binding energy 170.3kcal/mol at the FMO-HF/STO-3G level, which was 52.3kcal/mol higher than that of the smallest domain-receptor complex. These data provide the consideration of the backyard bulkiness beyond the binding site of protein to the protein-ligand stability.

Schon A., Madani N., Klein J. C., Hubicki A., Ng D., Yang X., Smith A. B., III, Sodroski J., and Freire E. (2006) Thermodynamics of binding of a low-molecular-weight CD4 mimetic to HIV-1 gp120. *Biochemistry* **45**, 10973-10980.

Abstract: NBD-556 and the chemically and structurally similar NBD-557 are two low-molecular weight compounds that reportedly block the interaction between the HIV-1 envelope glycoprotein gp120 and its receptor, CD4. NBD-556 binds to gp120 with a binding affinity of $2.7 \times 10^5 \text{ M}^{-1}$ ($K_d = 3.7 \text{ }\mu\text{M}$) in a process characterized by a large favorable change in enthalpy partially compensated by a large unfavorable entropy change, a thermodynamic signature similar to that observed for binding of sCD4 to gp120. NBD-556 binding is associated with a large structuring of the gp120 molecule, as also demonstrated by CD spectroscopy. NBD-556, like CD4, activates the binding of gp120 to the HIV-1 coreceptor, CCR5, and to the 17b monoclonal antibody, which recognizes the coreceptor binding site of gp120. NBD-556 stimulates HIV-1 infection of CD4-negative, CCR5-expressing cells. The thermodynamic signature of the binding of NBD-556 to gp120 is very different from that of another viral entry inhibitor, BMS-378806. Whereas NBD-556 binds gp120 with a large favorable enthalpy and compensating unfavorable entropy changes, BMS-378806 does so with a small binding enthalpy change in a mostly entropy-driven process. NBD-556 is a competitive inhibitor of sCD4 and elicits a similar structuring of the coreceptor binding site, whereas BMS-378806 does not compete with sCD4 and does not induce coreceptor binding. These studies demonstrate that low-molecular-weight compounds can induce conformational changes in the HIV-1 gp120 glycoprotein similar to those observed upon CD4 binding, revealing distinct strategies for inhibiting the function of the HIV-1 gp120 envelope glycoprotein. Furthermore, competitive and noncompetitive compounds have characteristic thermodynamic signatures that can be used to guide the design of more potent and effective viral entry inhibitors.

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.

Sharma V.A., Kan E., Sun Y., Lian Y., Cisto J., Frasca V., Hilt S., Stamatatos L., Donnelly J.J., Ulmer J.B., Barnett S.W., and Srivastava I.K. (2006) Structural characteristics correlate with immune responses induced by HIV envelope glycoprotein vaccines. *Virology* **352**, 131-144.

Abstract: HIV envelope glycoprotein (Env) is the target for inducing neutralizing antibodies. Env is present on the virus surface as a trimer, and, upon binding to CD4, a cascade of events leads to structural rearrangement exposing the co-receptor binding site and entry into the CD4+ host target cells. We have designed monomeric and trimeric Env constructs with and without deletion of the variable loop 2 (DeltaV2) from SF162, a subtype B primary isolate, and performed biophysical, biochemical and immunological studies to establish a potential structure-functional relationship. We expressed these Envs in CHO cells, purified the proteins to homogeneity and performed biophysical studies to define the binding properties to CD4, structural characteristics and exposure of epitopes recognized by b12 and CD4i mAb (17B) on both full-length and mutant HIV Env proteins. Parameters evaluated include oligomerization state, number and affinity of CD4 binding sites, enthalpy and entropy of the Env-CD4 interaction and affinity for b12 and 17b mAbs. We observed one CD4 binding site per monomer and three active CD4 binding sites per trimer. A 40-fold difference in affinity of the gp120 monomer vs. the o-gp140 trimer towards CD4 was observed ($K_d = 58$ nM and 1.5 nM, respectively), whereas only a 2-fold difference was observed for the V2 deleted Envs (K_d of gp120DeltaV2 = 19 nM, K_d of o-gp140DV2 = 9.3 nM). Monomers had 3-fold higher affinity to the mAb 17b and at least 3-fold weaker affinity to b12 compared to trimers, with gp120DV2 having the weakest affinity for b12 ($K_d = 446$ nM). Affinity of CD4 binding correlated with proportion of the antibodies induced against the conformational epitopes by the corresponding Envs, and changes in mAb binding correlated with the induction of antibodies directed against linear epitopes. Furthermore, biophysical analysis reveals that the V2 deletion has broad structural implications in the monomer not shared by the trimer, and these changes are reflected in the quality of the immune responses induced in rabbits. These data suggest that biophysical characteristics of HIV Env, such as affinity for CD4, and exposure of important neutralizing epitopes, such as those recognized by b12 mAb, may be important predictors of its in vivo efficacy and may serve as important surrogate markers for screening Env structures as potential vaccine candidates.

Shaw-Reid C. A., Munshi V., Graham P., Wolfe A., Witmer M., Danzeisen R., Olsen D. B., Carroll S. S., Embrey M., Wai J. S., Miller M. D., Cole J. L., and Hazuda D. J. (2003) Inhibition of HIV-1 ribonuclease H by a novel diketo acid, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid. *J Biol Chem* **278**, 2777-2780.

Abstract: Human immunodeficiency virus-type 1 (HIV-1) reverse transcriptase (RT) coordinates DNA polymerization and ribonuclease H (RNase H) activities using two discrete active sites embedded within a single heterodimeric polyprotein. We have identified a novel thiophene diketo acid, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid, that selectively inhibits polymerase-independent RNase H cleavage ($IC(50) = 3.2$ microm) but has no effect on DNA polymerization ($IC(50) > 50$ microm). The activity profile of the diketo acid is shown to be distinct from previously described compounds, including the polymerase inhibitor foscarnet and the putative RNase H inhibitor 4-chlorophenylhydrazone. Both foscarnet and the hydrazone inhibit RNase H cleavage and DNA polymerization activities of RT, yet neither inhibits the RNase H activity of RT containing a mutation in the polymerase active site (D185N) or an isolated HIV-1 RNase H domain chimera containing the alpha-C helix from Escherichia coli RNase HI, suggesting these compounds affect RNase H indirectly. In contrast, the diketo acid inhibits the RNase H activity of the isolated RNase H domain as well as full-length RT, and inhibition is not affected by the polymerase active site mutation. In isothermal titration calorimetry studies using the isolated RNase H domain, binding of the diketo acid is independent of nucleic acid but strictly requires $Mn(2+)$ implying a direct interaction between the inhibitor and the RNase H active site. These studies demonstrate that inhibition of HIV-1 RNase H may occur by either direct or indirect mechanisms, and they provide a framework for identifying novel agents such as 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid that specifically targets RNase H.

Shenoy S. R., O'Keefe B. R., Bolmstedt A. J., Cartner L. K., and Boyd M. R. (2001) Selective interactions of the human immunodeficiency virus-inactivating protein cyanovirin-N with high-mannose oligosaccharides on gp120 and other glycoproteins. *J Pharmacol Exp Ther* **297**, 704-710.

Abstract: The virucidal protein cyanovirin-N (CV-N) mediates its highly potent anti-human immunodeficiency virus activity, at least in part, through interactions with the viral envelope glycoprotein gp120. Here we dissect in further detail the mechanism of CV-N's glycosylation-dependent binding to gp120. Isothermal titration calorimetry (ITC) binding studies of CV-N with endoglycosidase H-treated gp120 showed that binding was completely abrogated by removal of high-mannose oligosaccharides from the glycoprotein. Additional ITC and circular dichroism spectral studies with CV-N and other glycoproteins as well showed that CV-N discriminately bound only glycoproteins that contain high-mannose oligosaccharides. Binding experiments with RNase B indicated that the single high-mannose oligosaccharide on that enzyme mediated all of its binding with CV-N ($K_d = 0.602 \mu\text{M}$). A finer level of oligosaccharide selectivity of CV-N was revealed in affinity chromatography-liquid chromatography-mass spectrometry experiments, which showed that CV-N preferentially bound only oligomannose-8 (Man-8) and oligomannose-9 isoforms of RNase B. Finally, we biophysically characterized the interaction of CV-N with a purified, single oligosaccharide, Man-8. The binding affinity of Man-8 for CV-N is unusually strong ($K_d = 0.488 \mu\text{M}$), several hundredfold greater than observed for oligosaccharides and their protein lectins ($K_d = 1 \mu\text{M}$ –1 mM), further establishing a critical role of high-mannose oligosaccharides in CV-N binding to glycoproteins.

Sia S. K., Carr P. A., Cochran A. G., Malashkevich V. N., and Kim P. S. (2002) Short constrained peptides that inhibit HIV-1 entry. *Proc Natl Acad Sci U S A* **99**, 14664-14669.

Abstract: Peptides corresponding to the C-terminal heptad repeat of HIV-1 gp41 (C-peptides) are potent inhibitors of HIV-1 entry into cells. Their mechanism of inhibition involves binding in a helical conformation to the central coiled coil of HIV-1 gp41 in a dominant-negative manner. Short C-peptides, however, have low binding affinity for gp41 and poor inhibitory activity, which creates an obstacle to the development of small drug-like C-peptides. To improve the inhibitory potency of short C-peptides that target the hydrophobic pocket region of gp41, we use two strategies to stabilize the C-peptide helix: chemical crosslinking and substitution with unnatural helix-favoring amino acids. In this study, the short linear peptide shows no significant inhibitory activity, but a constrained peptide (C14linkmid) inhibits cell-cell fusion at micromolar potency. Structural studies confirm that the constrained peptides bind to the gp41 hydrophobic pocket. Calorimetry reveals that, of the peptides analyzed, the most potent are those that best balance the changes in binding enthalpy and entropy, and surprisingly not those with the highest helical propensity as measured by circular dichroism spectroscopy. Our study reveals the thermodynamic basis of inhibition of an HIV C-peptide, demonstrates the utility of constraining methods for a short antiviral peptide inhibitor, and has implications for the future design of constrained peptides.

Singh M., Krajewski M., Mikolajka A., and Holak T. A. (2005) Molecular determinants for the complex formation between the retinoblastoma protein and LXCXE sequences. *J Biol Chem* **280**, 37868-37876.

Abstract: The retinoblastoma tumor suppressor protein (pRb) is a key negative regulator of cell proliferation that is frequently dysregulated in human cancer. Many viral oncoproteins (for example, HPV E7 and E1A) are known to bind to the pRb pocket domain via a LXCXE binding motif. There are also some 20 cellular proteins that contain a LXCXE motif and have been reported to associate with the pocket domain of pRb. Using NMR spectroscopy and isothermal calorimetry titration, we show that LXCXE peptides of viral oncoproteins bind strongly to the pocket domain of pRb. Additionally, we show that LXCXE-like peptides of HDAC1 bind to the same site on pRb with a weak (micromolar) and transient association. Systematic substitution of residues other than conserved Leu, Cys, and Glu show that the residues flanking the LXCXE are important for the binding, whereas positively charged amino acids in the XLXCXXXX sequence significantly weaken the interaction.

Smith R. F., Freyer M. W. and Lewis E. A. (2007) Biophysical characterization of vaccinia virus thymidine kinase substrate utilization. *J Virol. Methods* **142**, 151-158.

Abstract: To provide information for the development of new antiviral compounds that inhibit orthopoxviruses, further characterization of the kinetics and thermodynamics that underlie substrate utilization reactions of vaccinia virus thymidine kinase (VVTK) has been undertaken. The kinetics of 2'deoxythymidine phosphorylation by VVTK and the thermodynamics of complex formation between

VVTK and the substrate 2' deoxythymidine were determined using spectroscopic and calorimetric techniques. These studies demonstrated that kinetic parameters for 2' deoxythymidine phosphorylation by VVTK were 25 μM and 0.2 s⁻¹ for $K(m)$ and $k(\text{cat})$, respectively. The enthalpy change, ΔH , for the enzyme catalyzed reaction is -18.1 kcal/mol. Thermodynamic studies for the formation of the enzyme substrate complex demonstrated a binding affinity ($K(a)$) of $4 \times 10^4 \text{M}^{-1}$, an enthalpy change for binding (ΔH) of -17.4 kcal/mol, and a reaction stoichiometry of two molecules of substrate binding to each enzyme tetramer. Kinetic and thermodynamic data were in agreement ($K(a)$ approximately $1/K(m)$) and showed similarities to literature values reported for herpes simplex virus thymidine kinase (HSV-TK) and human thymidine kinase 1 (hTK1) with respect to $k(\text{cat})$ but not with respect to $K(m)$. The $K(m)$ value found for VVTK in this study is nearly two orders of magnitude larger than the values reported for the hTK1 and the HSV TK enzymes.

Song J., Zhang Z., Hu W., and Chen Y. (2005) Small ubiquitin-like modifier (SUMO) recognition of a SUMO binding motif: a reversal of the bound orientation. *J Biol Chem.* **280**, 40122-9.

Abstract: Sumoylation has recently been identified as an important mechanism that regulates protein interactions and localization in essential cellular functions, such as gene transcription, subnuclear structure formation, viral infection, and cell cycle progression. A SUMO binding amino acid sequence motif (SBM), which recognizes the SUMO moiety of modified proteins in sumoylation-dependent cellular functions, has been consistently identified by several recent studies. To understand the mechanism of SUMO recognition by the SBM, we have solved the solution structure of SUMO-1 in complex with a peptide containing the SBM derived from the protein PIASX (KVDVIDLTIESSSDEEEDPPAKR). Surprisingly, the structure reveals that the bound orientation of the SBM can reverse depending on the sequence context. The structure also reveals a novel mechanism of recognizing target sequences by a ubiquitin-like module. Unlike ubiquitin binding motifs, which all form helices and bind to the main beta-sheet of ubiquitin, the SBM forms an extended structure that binds between the alpha-helix and a beta-strand of SUMO-1. This study provides a clear mechanism of the SBM sequence variations and its recognition of the SUMO moiety in sumoylated proteins.

Srivastava I. K., Kan E., Sun Y., Sharma V. A., Cisto J., Burke B., Lian Y., Hilt S., Biron Z., Hartog K., Stamatatos L., Cheng R. H., Ulmer J. B. and Barnett S. W. (2008) Comparative evaluation of trimeric envelope glycoproteins derived from subtype C and B HIV-1 R5 isolates. *Virology* **372**, 273-290.

Abstract: We previously reported that an envelope (Env) glycoprotein immunogen (o-gp140DeltaV2SF162) containing a partial deletion in the second variable loop (V2) derived from the R5-tropic HIV-1 isolate SF162 partially protected vaccinated rhesus macaques against pathogenic SHIV(SF162P4) virus. Extending our studies to subtype C isolate TV1, we have purified o-gp140DeltaV2TV1 (subtype C DeltaV2 trimer) to homogeneity, performed glycosylation analysis, and determined its ability to bind CD4, as well as a panel of well-characterized neutralizing monoclonal antibodies (mAb). In general, critical epitopes are preserved on the subtype C DeltaV2 trimer; however, we did not observe significant binding for the b12 mAb. The molecular mass of subtype C DeltaV2 trimer was found to be 450 kDa, and the hydrodynamic radius was found to be 10.87 nm. Our data suggest that subtype C DeltaV2 trimer binds to CD4 with an affinity comparable to o-gp140DeltaV2SF162 (subtype B DeltaV2 trimer). Using isothermal titration calorimetric (ITC) analysis, we demonstrated that all three CD4 binding sites (CD4-BS) in both subtype C and B trimers are exposed and accessible. However, compared to subtype B trimer, the three CD4-BS in subtype C trimer have different affinities for CD4, suggesting a cooperativity of CD4 binding in subtype C trimer but not in subtype B trimer. Negative staining electron microscopy of the subtype C DeltaV2 trimer has demonstrated that it is in fact a trimer. These results highlight the importance of studying subtype C Env, and also of developing appropriate subtype C-specific reagents that may be used for better immunological characterization of subtype C Env for developing an AIDS vaccine

Strauss D. M. and Wuttke D. S. (2007) Characterization of protein-protein interactions critical for poliovirus replication: analysis of 3AB and VPg binding to the RNA-dependent RNA polymerase. *J Virol.* **81**, 6369-6378.

Abstract: Two critical interactions within the poliovirus RNA replication complex are those of the RNA-dependent RNA polymerase 3D with the viral proteins 3AB and VPg. 3AB is a membrane-binding protein responsible for the localization of the polymerase to the membranous vesicles at which replication occurs.

VPg (a peptide comprising the 3B region of 3AB) is the 22-residue soluble product of 3AB cleavage and serves as the protein primer for RNA replication. The detailed interactions of these proteins with the RNA-dependent RNA polymerase 3D were analyzed to elucidate the precise roles of 3AB and VPg in the viral RNA replication complex. Using a membrane-based pull-down assay, we have identified a binding "hot-spot" spanning residues 100 to 104 in the 3B (VPg) region of 3AB which plays a critical role in mediating the interaction of 3AB with the polymerase. Isothermal titration calorimetry shows that the interaction of VPg with 3D is enthalpically driven, with a dissociation constant of 11 μM . Mutational analyses of VPg indicate that a subset of the residues important for 3AB-3D binding are also important for VPg-3D binding. Two residues in particular, P14 and R17, were shown to be absolutely critical for the binding interaction. This work provides the direct characterization of two binding interactions critical for the replication of this important class of viruses and identifies a conserved polymerase binding sequence responsible for targeting the polymerase.

Surleraux D. L., de Kock H. A., Verschueren W. G., Pille G. M., Maes L. J., Peeters A., Vendeville S., De Meyer S., Azijn H., Pauwels R., de Bethune M. P., King N. M., Prabu-Jeyabalan M., Schiffer C. A., and Wigerinck P. B. (2005) Design of HIV-1 protease inhibitors active on multidrug-resistant virus. *J Med Chem* **48**, 1965-1973.

Abstract: On the basis of structural data gathered during our ongoing HIV-1 protease inhibitors program, from which our clinical candidate TMC114 9 was selected, we have discovered new series of fused heteroaromatic sulfonamides. The further extension into the P2' region was aimed at identifying new classes of compounds with an improved broad spectrum activity and acceptable pharmacokinetic properties. Several of these compounds display an exceptional broad spectrum activity against a panel of highly cross-resistant mutants. Certain members of these series exhibit favorable pharmacokinetic profiles in rat and dog. Crystal structures and molecular modeling were used to rationalize the broad spectrum profile resulting from the extension into the P2' pocket of the HIV-1 protease.

Tang K. F., Abdullah M. P., Yusoff K. and Tan W. S. (2007) Interactions of hepatitis B core antigen and peptide inhibitors. *J Med. Chem* **50**, 5620-5626.

Abstract: The core protein (HBcAg) of hepatitis B virus (HBV) has been shown to interact with the large surface antigen during HBV morphogenesis, and these interactions can be blocked by small peptides selected from either linear or constrained phage display peptide libraries. The association of HBcAg with peptide inhibitors was quantitatively evaluated by isothermal titration calorimetry. The thermodynamic data show that the interaction between HBcAg and peptide MHRSLGMRMKGGA is enthalpy-driven and occurs at a 3:1 stoichiometry and dissociation constant (K_d) value of 79.4 μM . However, peptide WSFFSNI displays a higher binding affinity for HBcAg with a K_d value of 18.5 μM when compared to peptide MHRSLGMRMKGGA. A combinatorial approach using chemical cross-linking and surface-enhanced laser desorption/ionization-time-of-flight-mass spectrometry shows that the Lys of peptide MHRSLGMRMKGGA interacted either with D64, E77, or D78 of HBcAg.

Todd M. J., Luque I., Velazquez-Campoy A., and Freire E. (2000) Thermodynamic basis of resistance to HIV-1 protease inhibition: calorimetric analysis of the V82F/I84V active site resistant mutant. *Biochemistry* **39**, 11876-11883.

Abstract: One of the most serious side effects associated with the therapy of HIV-1 infection is the appearance of viral strains that exhibit resistance to protease inhibitors. The active site mutant V82F/I84V has been shown to lower the binding affinity of protease inhibitors in clinical use. To identify the origin of this effect, we have investigated the binding thermodynamics of the protease inhibitors indinavir, ritonavir, saquinavir, and nelfinavir to the wild-type HIV-1 protease and to the V82F/I84V resistant mutant. The main driving force for the binding of all four inhibitors is a large positive entropy change originating from the burial of a significant hydrophobic surface upon binding. At 25 degrees C, the binding enthalpy is unfavorable for all inhibitors except ritonavir, for which it is slightly favorable (-2.3 kcal/mol). Since the inhibitors are pre-shaped to the geometry of the binding site, their conformational entropy loss upon binding is small, a property that contributes to their high binding affinity. The V82F/I84V active site mutation lowers the affinity of the inhibitors by making the binding enthalpy more positive and making the entropy change slightly less favorable. The effect on the enthalpy change is, however, the major one. The predominantly enthalpic effect of the V82F/I84V mutation is consistent with the idea that the introduction of the bulkier Phe side chain at position 82 and the Val side chain at position 84 distort the binding site and

weaken van der Waals and other favorable interactions with inhibitors preshaped to the wild-type binding site. Another contribution of the V82F/I84V to binding affinity originates from an increase in the energy penalty associated with the conformational change of the protease upon binding. The V82F/I84V mutant is structurally more stable than the wild-type protease by about 1.4 kcal/mol. This effect, however, affects equally the binding affinity of substrate and inhibitors.

Turner D. C., Straume M., Kasimova M. R., and Gaber B. P. (1995) Thermodynamics of interaction of the fusion-inhibiting peptide Z-D-Phe-L-Phe-Gly with dioleoylphosphatidylcholine vesicles: direct calorimetric determination. *Biochemistry* **34**, 9517-9525.

Abstract: The binding of the fusion-inhibiting peptide Z-D-Phe-L-Phe-Gly to unilamellar lipid vesicles of dioleoylphosphatidylcholine (DOPC) was investigated by isothermal titration calorimetry (ITC). The peptide Z-D-Phe-L-Phe-Gly is known to inhibit fusion of myxo- and paramyxoviruses with cells as well as cell-cell and vesicle-vesicle fusion in model systems. Calorimetric titrations conducted over a range of temperatures permitted characterization of the thermodynamics of the interaction of Z-D-Phe-L-Phe-Gly with model DOPC lipid membranes. Simultaneous global analysis of 15 ITC binding curves acquired at four different temperatures allowed determination of the equilibrium site association constant (K), stoichiometry of binding (n), binding enthalpy change (ΔH), and heat capacity change of binding (ΔC_p) in a single set of experiments. The binding affinity and enthalpy change per mole of DOPC bound at 25 degrees C was $\log K = 2.463 \pm 0.075$ and $\Delta H = -1.07 \pm 0.12$ kcal/mol DOPC while the binding heat capacity change per mole of DOPC bound was $\Delta C_p = -20.3 \pm 2.8$ cal/(K.mol DOPC) with a temperature dependence (from 10-45 degrees C) of $d(\Delta C_p)/dT = 0.37 \pm 0.18$ cal/(K².mol DOPC). A temperature-independent binding stoichiometry was determined to be $n = 5.56 \pm 0.33$ DOPC molecules per Z-D-Phe-L-Phe-Gly. A comparison of these results with previous peptide-lipid binding studies is discussed as is their relevance to a current model of the interaction of fusion-inhibiting peptides with phospholipid membranes.

Velazquez-Campoy A., Todd M. J., Vega S., and Freire E. (2001) Catalytic efficiency and vitality of HIV-1 proteases from African viral subtypes. *Proc Natl Acad Sci U S A* **98**, 6062-6067.

Abstract: The vast majority of HIV-1 infections in Africa are caused by the A and C viral subtypes rather than the B subtype prevalent in the United States and Western Europe. Genomic differences between subtypes give rise to sequence variations in the encoded proteins, including the HIV-1 protease. Because some amino acid polymorphisms occur at sites that have been associated with drug resistance in the B subtype, it is important to assess the effectiveness of protease inhibitors that have been developed against different subtypes. Here we report the enzymatic characterization of HIV-1 proteases with sequences found in drug-naïve Ugandan adults. The A protease used in these studies differs in seven positions (I13V/E35D/M36I/R41K/R57K/H69K/L89M) in relation to the consensus B subtype protease. Another protease containing a subset of these amino acid polymorphisms (M36I/R41K/H69K/L89M), which are found in subtype C and other HIV subtypes, also was studied. Both proteases were found to have similar catalytic constants, k_{cat} , as the B subtype. The C subtype protease displayed lower K_m values against two different substrates resulting in a higher (2.4-fold) catalytic efficiency than the B subtype protease. Indinavir, ritonavir, saquinavir, and nelfinavir inhibit the A and C subtype proteases with 2.5-7-fold and 2-4.5-fold weaker K_i s than the B subtype. When all factors are taken into consideration it is found that the C subtype protease has the highest vitality (4-11 higher than the B subtype) whereas the A subtype protease exhibits values ranging between 1.5 and 5. These results point to a higher biochemical fitness of the A and C proteases in the presence of existing inhibitors.

Velazquez-Campoy A., Vega S., and Freire E. (2002) Amplification of the effects of drug resistance mutations by background polymorphisms in HIV-1 protease from African subtypes. *Biochemistry* **41**, 8613-8619.

Abstract: The vast majority of HIV-1 infections worldwide are caused by the C and A viral subtypes rather than the B subtype prevalent in the United States and Western Europe. Genomic differences between subtypes give rise to sequence variations in the encoded proteins, including those identified as targets for antiretroviral therapies. In the case of the HIV-1 protease, we reported earlier [Velazquez-Campoy et al. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 6062-6067] that proteases from the C and A subtypes exhibit a higher biochemical fitness in the presence of widely prescribed protease inhibitors. In this paper we present a complete thermodynamic dissection of the differences between proteases from different subtypes and the effects of the V82F/I84V drug-resistant mutation within the framework of the B, C, and A subtypes. These

studies involved four inhibitors in clinical use (indinavir, saquinavir, ritonavir, and nelfinavir) and a second-generation protease inhibitor (KNI-764). Naturally occurring amino acid polymorphisms found in proteases from the C and A subtypes lower the binding affinities of existing clinical inhibitors by factors ranging between 2 and 7.5 which by themselves are not enough to cause drug resistance. The preexisting lower affinity in the C and A subtypes, however, significantly amplifies the effects of the drug-resistant mutation. Relative to the wild-type B subtype protease, the V82F/I84V drug-resistant mutation within the C and A subtypes lowers the binding affinity of inhibitors by factors ranging between 40 and 3000. When the enzyme kinetic properties (k_{cat} and K_m) are included in the analysis, the biochemical fitness of the C and A subtype drug-resistant mutants can be up to 1000-fold higher than that of the wild-type B subtype protease in the presence of the studied inhibitors. From a thermodynamic standpoint, the combined effects of the drug-resistant mutations and the natural amino acid polymorphisms on the Gibbs energy are additive and involve significant alterations in the enthalpy and entropy changes associated with inhibitor binding. At the biochemical level, the combined effects of naturally existing polymorphisms and drug-resistant mutations might have important consequences on the long-term viability of current HIV-1 protease inhibitors.

Verkhivker G. M. (1996) Empirical free energy calculations of human immunodeficiency virus type 1 protease crystallographic complexes. II. Knowledge-based ligand-protein interaction potentials applied to thermodynamic analysis of hydrophobic mutations. *Pac Symp Biocomput* 638-652.

Abstract: Empirical free energy calculations of HIV-1 protease crystallographic complexes based on the developed knowledge-based ligand-protein interaction potentials have enabled a detailed thermodynamic analysis. Binding free energies are estimated within an empirical model that postulates that hydrophobic effect, mean field ligand-protein interaction potentials and conformational entropy changes are the dominant forces that determine complex formation. To provide a quantitative framework of the binding thermodynamics contributions the derived knowledge-based potentials have been linked with the hydrophobicity and conformational entropy scales originally developed to explain protein stability. The comparative analysis of studied inhibitors provides reasonable estimates of distinctions in their binding affinity with HIV-1 protease and gives insight into the nature of the binding determinants. The binding free energy changes upon a simple hydrophobic mutation Ile \rightarrow Val in the JG-365, MVT-101 and U75875 inhibitors of HIV-1 protease have been evaluated within a model that includes the effects of solvation, cavity formation, conformational entropy and mean field ligand-protein interactions. In general, free energy changes associated with a particular perturbation of a system can not be rigorously decomposed into separate terms from first principles. We explored the relationships between the changes in hydrophobic contributions and mean field ligand-protein interaction energies in the context of a totally buried and dense area of the binding site. We assume, therefore, that these simple hydrophobic deletions would not induce noticeable conformational changes in the enzyme and can be interpreted with some confidence in the framework of the model. The analysis has revealed the decisive effect of the energetics of ligand-protein interactions on the estimated free energy changes.

Vyas N. K., Vyas M. N., Chervenak M. C., Bundle D. R., Pinto B. M., and Quioco F. A. (2003) Structural basis of peptide-carbohydrate mimicry in an antibody-combining site. *Proc Natl Acad Sci U S A* **100**, 15023-15028.

Abstract: The structure of a complex between the Fab fragment of the antibody (SYA/J6) specific for the cell surface O-antigen polysaccharide of the pathogen *Shigella flexneri* Y and an octapeptide (Met-Asp-Trp-Asn-Met-His-Ala-Ala), a functional mimic of the O-antigen, has been determined at 1.8-Å resolution. Comparison of the structure with that of the complex with the pentasaccharide antigen [\rightarrow 2)- α -l-Rha-(1 \rightarrow 2)- α -l-Rha-(1 \rightarrow 3)- α -l-Rha-(1 \rightarrow 3)- β -d- GlcNAc-(1 \rightarrow 2)- α -l-Rha-(1 \rightarrow)] reveals the molecular recognition process by which a peptide mimics a carbohydrate in binding to an antibody. The binding modes of the two ligands differ considerably. Octapeptide binding complements the shape of the combining site groove much better than pentasaccharide binding. Moreover, the peptide makes a much greater number of contacts (126), which are mostly van der Waals interactions, with the Fab than the saccharide (74). An unusual feature is also the involvement of 12 water molecules in mediating hydrogen bonds between residues within the peptide or of the peptide and Fab. Despite better shape complementarity and greater number of contacts, the octapeptide binds with an affinity ($K_A = 2.5 \times 10^5 \text{ M}^{-1}$, measured by calorimetry) only approximately 2-fold tighter than the pentasaccharide. The structural results are relevant to the design of peptide mimetics with improved affinity for use as vaccines.

Wahid A. M., Coventry V. K. and Conn G. L. (2008) Systematic deletion of the adenovirus-associated RNAI terminal stem reveals a surprisingly active RNA inhibitor of double-stranded RNA-activated protein kinase. *J Biol Chem* **283**, 17485-17493.

Abstract: Adenoviruses use the short noncoding RNA transcript virus-associated (VA) RNA(I) to counteract two critical elements of the host cell defense system, innate cellular immunity and RNA interference, mediated by the double-stranded RNA-activated protein kinase (PKR) and Dicer/RNA-induced silencing complex, respectively. We progressively shortened the VA RNA(I) terminal stem to examine its necessity for inhibition of PKR. Each deletion, up to 15 bp into the terminal stem, resulted in a cumulative decrease in PKR inhibitory activity. Remarkably, however, despite significant apparent destabilization of the RNA structure, the final RNA mutant that lacked the entire terminal stem (TSDelta21 RNA) efficiently bound PKR and exhibited wild-type inhibitory activity. TSDelta21 RNA stability was strongly influenced by solution pH, indicating the involvement of a protonated base within the VA RNA(I) central domain tertiary structure. Gel filtration chromatography and isothermal titration calorimetry analysis indicated that wild-type VA RNA(I) and TSDelta21 RNA form similar 1:1 complexes with PKR but that the latter lacks secondary binding site(s) that might be provided by the terminal stem. Although TSDelta21 RNA bound PKR with wild-type $K(d)$, and overall change in free energy (ΔG), the thermodynamics of binding (ΔH and ΔS) were significantly altered. These results demonstrate that the VA RNA(I) terminal stem is entirely dispensable for inhibition of PKR. Potentially, VA RNA(I) is therefore a truly bi-functional RNA; Dicer processing of the VA RNA(I) terminal stem saturates the RNA interference system while generating a "mini-VA RNA(I)" molecule that remains fully active against PKR

Wang Q. Y., Dolmer K., Huang W., Gettins P. G., and Rong L. (2001) Role of calcium in protein folding and function of Tva, the receptor of subgroup A avian sarcoma and leukosis virus. *J Virol* **75**, 2051-2058.

Abstract: Tva is the cellular receptor for subgroup A avian sarcoma and leukosis virus (ASLV-A). The viral receptor function of Tva is determined by a 40-residue cysteine-rich motif called the LDL-A module. In this study, we expressed and purified the wild-type (wt) Tva LDL-A module as well as several mutants and examined their in vitro folding properties. We found that, as for other LDL-A modules, correct folding and structure of the Tva LDL-A module is Ca^{2+} dependent. When calcium was present during in vitro protein folding, the wt module was eluted as a single peak by reverse-phase high-pressure liquid chromatography. Furthermore, two-dimensional nuclear magnetic resonance (NMR) spectroscopy gave well-dispersed spectra in the presence of calcium. In contrast, the same protein folded in vitro in the absence of calcium was eluted as multiple broad peaks and gave a poorly dispersed NMR spectrum in the presence of calcium. The calcium affinity (K_d) of the Tva LDL-A module, determined by isothermal titration calorimetry, is approximately 40 μM . Characterization of several Tva mutants provided further evidence that calcium is important in protein folding and function of Tva. Mutations of the Ca^{2+} -binding residues (D46A and E47A) completely abrogated the Ca^{2+} -binding ability of Tva, and the proteins were not correctly folded. Interestingly, mutations of two non-calcium-binding residues (W48A and L34A) also exerted adverse effect on Ca^{2+} -dependent folding, albeit to a much less extent. Our results provide new insights regarding the structure and function of Tva in ASLV-A entry.

White P. W., Titolo S., Brault K., Thauvette L., Pelletier A., Welchner E., Bourgon L., Doyon L., Ogilvie W. W., Yoakim C., Cordingley M. G., and Archambault J. (2003) Inhibition of human papillomavirus DNA replication by small molecule antagonists of the E1-E2 protein interaction. *J Biol Chem* **278**, 26765-26772.

Abstract: Human papillomavirus (HPV) DNA replication is initiated by recruitment of the E1 helicase by the E2 protein to the viral origin. Screening of our corporate compound collection with an assay measuring the cooperative binding of E1 and E2 to the origin identified a class of small molecule inhibitors of the protein interaction between E1 and E2. Isothermal titration calorimetry and changes in protein fluorescence showed that the inhibitors bind to the transactivation domain of E2, the region that interacts with E1. These compounds inhibit E2 of the low risk HPV types 6 and 11 but not those of high risk HPV types or of cottontail rabbit papillomavirus. Functional evidence that the transactivation domain is the target of inhibition was obtained by swapping this domain between a sensitive (HPV11) and a resistant (cottontail rabbit papillomavirus) E2 type and by identifying an amino acid substitution, E100A, that increases inhibition by approximately 10-fold. This class of inhibitors was found to antagonize specifically the E1-E2 interaction in vivo and to inhibit HPV DNA replication in transiently transfected cells. These results highlight the potential of the E1-E2 interaction as a small molecule antiviral target.

Willcox B. E., Gao G. F., Wyer J. R., O'Callaghan C. A., Boulter J. M., Jones E. Y., van der Merwe P. A., Bell J. I., and Jakobsen B. K. (1999) Production of soluble alphabeta T-cell receptor heterodimers suitable for biophysical analysis of ligand binding. *Protein Sci* **8**, 2418-2423.

Abstract: A method to produce alphabeta T-cell receptors (TCRs) in a soluble form suitable for biophysical analysis was devised involving in vitro refolding of a TCR fusion protein. Polypeptides corresponding to the variable and constant domains of each chain of a human and a murine receptor, fused to a coiled coil heterodimerization motif from either c-Jun (alpha) or v-Fos (beta), were overexpressed separately in *Escherichia coli*. Following recovery from inclusion bodies, the two chains of each receptor were denatured, and then refolded together in the presence of denaturants. For the human receptor, which is specific for the immunodominant influenza A HLA-A2-restricted matrix epitope (M58-66), a heterodimeric protein was purified in milligram yields and found to be homogeneous, monomeric, antibody-reactive, and stable at concentrations lower than 1 μ M. Using similar procedures, analogous results were obtained with a murine receptor specific for an influenza nucleoprotein epitope (366-374) restricted by H2-Db. Production of these receptors has facilitated a detailed analysis of viral peptide-Major Histocompatibility Complex (peptide-MHC) engagement by the TCR using both surface plasmon resonance (SPR) and, in the case of the human TCR, isothermal titration calorimetry (ITC) (Willcox et al., 1999). The recombinant methods described should enable a wide range of TCR-peptide-MHC interactions to be studied and may also have implications for the production of other heterodimeric receptor molecules.

Xiao B., Spencer J., Clements A., Ali-Khan N., Mittnacht S., Broceno C., Burghammer M., Perrakis A., Marmorstein R., and Gamblin S. J. (2003) Crystal structure of the retinoblastoma tumor suppressor protein bound to E2F and the molecular basis of its regulation. *Proc Natl Acad Sci U S A* **100**, 2363-2368.

Abstract: The retinoblastoma tumor suppressor protein (pRb) regulates the cell cycle, facilitates differentiation, and restrains apoptosis. Furthermore, dysfunctional pRb is thought to be involved in the development of most human malignancies. Many of the functions of pRb are mediated by its regulation of the E2F transcription factors. To understand the structural basis for this regulation, we have determined the crystal structure of a fragment of E2F in complex with the pocket domain of the tumor suppressor protein. The pRb pocket, comprising the A and B cyclin-like domains, is the major focus of tumorigenic mutations in the protein. The fragment of E2F used in our structural studies, residues 409-426 of E2F-1, represents the core of the pRb-binding region of the transcription factor. The structure shows that E2F binds at the interface of the A and B domains of the pocket making extensive interactions with conserved residues from both. We show by solution studies that a second site, probably contained within the "marked box" region of E2F, is responsible for additional interactions with the pRb pocket but is insufficient for complex formation on its own. In addition, we show that the interaction of the core binding fragment of E2F with pRb is inhibited by phosphorylation of the tumor suppressor protein by CDK2cyclin DE. Finally, our data reveal that the tight binding of the human papillomavirus E7 oncoprotein to pRb prevents subsequent interactions with the marked box region of E2F but not with its core binding region.

Yakubovskaya E., Chen Z., Carrodegua J.A., Kisker C., Bogenhagen D.F. (2006) Functional human mitochondrial DNA polymerase gamma forms a heterotrimer. *J Biol Chem*. **281**, 374-82.

Abstract: Mitochondrial DNA polymerase gamma (pol gamma) is responsible for replication and repair of mtDNA and is mutated in individuals with genetic disorders such as chronic external ophthalmoplegia and Alpers syndrome. pol gamma is also an adventitious target for toxic side effects of several antiviral compounds, and mutation of its proofreading exonuclease leads to accelerated aging in mouse models. We have used a variety of physical and functional approaches to study the interaction of the human pol gamma catalytic subunit with both the wild-type accessory factor, pol gammaB, and a deletion derivative that is unable to dimerize and consequently is impaired in its ability to stimulate processive DNA synthesis. Our studies clearly showed that the functional human holoenzyme contains two subunits of the processivity factor and one catalytic subunit, thereby forming a heterotrimer. The structure of pol gamma seems to be variable, ranging from a single catalytic subunit in yeast to a heterodimer in *Drosophila* and a heterotrimer in mammals.

Yanchunas J., Jr., Langley D. R., Tao L., Rose R. E., Friberg J., Colonna R. J., and Doyle M. L. (2005) Molecular basis for increased susceptibility of isolates with atazanavir resistance-conferring substitution I50L to other protease inhibitors. *Antimicrob Agents Chemother* **49**, 3825-3832.

Abstract: Protease inhibitors (PIs) are highly effective drugs against the human immunodeficiency virus (HIV), yet long-term therapeutic use is limited by emergence of HIV type 1 (HIV-1) protease substitutions that confer cross-resistance to multiple protease inhibitor drugs. Atazanavir is a highly potent HIV protease inhibitor with a distinct resistance profile that includes effectiveness against most HIV-1 isolates resistant to one or two PIs. The signature resistance substitution for atazanavir is I50L, and it is frequently (53%) accompanied by a compensatory A71V substitution that helps restore viability and increases atazanavir resistance levels. We measured the binding affinities of wild-type (WT) and I50L/A71V HIV-1 proteases to atazanavir and other currently approved PIs (ritonavir, lopinavir, saquinavir, nelfinavir, indinavir, and amprenavir) by isothermal titration calorimetry. Remarkably, we find that all of the PIs have 2- to 10-fold increased affinities for I50L/A71V protease, except for atazanavir. The results are also manifested by thermal stability measures of affinity for WT and I50L/A71V proteases. Additional biophysical and enzyme kinetics experiments show I50L/A71V protease is a stable enzyme with catalytic activity that is slightly reduced (34%) relative to the WT. Computational modeling reveals that the unique resistance phenotype of I50L/A71V protease likely originates from bulky tert-butyl groups at P2 and P2' (specific to atazanavir) that sterically clash with methyl groups on residue L50. The results of this study provide a molecular understanding of the novel hypersusceptibility of atazanavir-resistant I50L/A71V-containing clinical isolates to other currently approved PIs.

Ye H. and Wu H. (2000) Thermodynamic characterization of the interaction between TRAF2 and tumor necrosis factor receptor peptides by isothermal titration calorimetry. *Proc Natl Acad Sci U S A* **97**, 8961-8966.

Abstract: The tumor necrosis factor receptor (TNFR) superfamily can induce diverse biological effects, including cell survival, proliferation, differentiation, and apoptosis. The major signal transducers for TNFRs are the family of TNF receptor associated factors (TRAFs). The direct interaction between TRAFs and the intracellular tails of TNFRs is the first step of this signal relay process. Structural studies have revealed a trimeric nature of TRAF2 and a symmetrical mode of receptor binding, suggesting the involvement of trivalent TNFR2-receptor interaction in the signal transduction. In this study, using isothermal titration calorimetry (ITC), we report thermodynamic characterization of the interaction between TRAF2 and monomeric peptide sequences from TNFR members, including TNFR2, CD40, CD30, O_x40, and 4-1BB, and the Epstein-Barr virus (EBV)-transforming protein, latent infection membrane protein-1 (LMP1). The dissociation constants of the interaction were shown to range between 40 μ M and 1.9 mM, which are substantially weaker than most protein-peptide interactions. The interaction is entirely driven by exothermic enthalpy, consistent with the abundance of polar contacts. The enthalpy of the interaction has a significant temperature dependence ($\Delta C_p = -245$ cal/mol small middle dotK). The unfavorable entropy in the interaction and the comparison with structural energetics calculations suggest the involvement of conformational rearrangement in the interaction. The low affinity of TRAF2 to monomeric receptor peptides further supports the importance of avidity contribution in TRAF2 recruitment by these receptors upon ligand-induced trimerization or higher order oligomerization.

Yoo S., Myszka D. G., Yeh C., McMurray M., Hill C. P., and Sundquist W. I. (1997) Molecular recognition in the HIV-1 capsid/cyclophilin A complex. *J Mol Biol* **269**, 780-795.

Abstract: The HIV-1 capsid protein (CA) makes an essential interaction with the human peptidyl prolyl isomerase, cyclophilin A (CypA), that results in packaging of CypA into the virion at a CA to CypA stoichiometry of approximately 10:1. The 231 amino acid residue capsid protein is composed of an amino-terminal CypA binding domain (1 to approximately 151; CA151) and a carboxyl-terminal dimerization domain (approximately 151 to 231). We find that CypA binds dimeric CA and monomeric CA151 with identical intrinsic affinities ($K_d = 16(+/-4)$ μ M). This result demonstrates that capsid dimerization and cyclophilin A binding are not thermodynamically coupled and suggests that the substoichiometric ratio of CypA in the HIV-1 virion results from the intrinsic stability of the CA/CypA complex. In the known co-crystal structure of the CA151/CypA complex, CypA binding is mediated exclusively by an exposed capsid loop that spans residues Pro85 to Pro93. The energetic contributions to CypA binding were quantified for each residue in this loop, and the results demonstrate that the Gly89-Pro90 dipeptide is the primary cyclophilin A recognition motif, with Pro85, Val86, His87, Ala88, and Pro93 also making energetically favorable contacts. These studies reveal that the active site of CypA, which can catalyze the isomerization of proline residues in vitro, also functions as a sequence-specific, protein-binding motif in HIV-1 replication.

Ziegler A., Blatter X. L., Seelig A., and Seelig J. (2003) Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis. *Biochemistry* **42**, 9185-9194.

Abstract: Cell-penetrating peptides (CPPs) traverse cell membranes of cultured cells very efficiently by a mechanism not yet identified. Recent theories for the translocation suggest either the binding of the CPPs to extracellular glycosaminoglycans or the formation of inverted micelles with negatively charged lipids. In the present study, the binding of the protein transduction domains (PTD) of human (HIV-1) and simian immunodeficiency virus (SIV) TAT peptide (amino acid residues 47-57, electric charge $z(p) = +8$) to membranes containing various proportions of negatively charged lipid (POPG) is characterized. Monolayer expansion measurements demonstrate that TAT-PTD insertion between lipids requires loosely packed monolayer films. For densely packed monolayers ($\pi > 29$ mN/m) and lipid bilayers, no insertion is possible, and binding occurs via electrostatic adsorption to the membrane surface. Light scattering experiments show an aggregation of anionic lipid vesicles when the electric surface charge is neutralized by TAT-PTD, the observed stoichiometry being close to the theoretical value of 1:8. Membrane binding was quantitated with isothermal titration calorimetry and three further methods. The reaction enthalpy is ΔH degrees approximately equal to -1.5 kcal/mol peptide and is almost temperature-independent with $\Delta C(p)$ degrees approximately 0 kcal/(mol K), indicating equal contributions of polar and hydrophobic interactions to the reaction heat capacity. The binding of TAT-PTD to the anionic membrane is described by an electrostatic attraction/chemical partition model. The electrostatic attraction energy, calculated with the Gouy-Chapman theory, accounts for approximately 80% of the binding energy. The overall binding constant, $K(\text{app})$, is approximately 10^3 - 10^4 M^{-1} . The intrinsic binding constant (K_p), corrected for electrostatic effects and describing the partitioning of the peptide between the lipid-water interface and the membrane, is small and is K_p approximately 1-10 M^{-1} . Deuterium and phosphorus-31 nuclear magnetic resonance demonstrate that the lipid bilayer remains intact upon TAT-PTD binding. The NMR data provide no evidence for nonbilayer structures and also not for domain formation. This is further supported by the absence of dye efflux from single-walled lipid vesicles. The electrostatic interaction between TAT-PTD and anionic phosphatidylglycerol is strong enough to induce a change in the headgroup conformation of the anionic lipid, indicating a short-lived but distinct correlation between the TAT-PTD and the anionic lipids on the membrane outside. TAT-PTD has a much lower affinity for lipid membranes than for glycosaminoglycans, making the latter interaction a more probable pathway for CPP binding to biological membranes.

Ziolkowska N. E., Shenoy S. R., O'Keefe B. R. and Wlodawer A. (2007) Crystallographic studies of the complexes of antiviral protein griffithsin with glucose and N-acetylglucosamine. *Protein Sci* **16**, 1485-1489.

Abstract: Crystal structures of complexes of an antiviral lectin griffithsin (GRFT) with glucose and N-acetylglucosamine were solved and refined at high resolution. In both complexes, all six monosaccharide-binding sites of GRFT were occupied and the mode of binding was similar to that of mannose. In our previous attempts to obtain a complex with N-acetylglucosamine by soaking, only a single site was occupied; thus, cocrystallization was clearly superior despite lower concentration of the ligand. Isothermal titration calorimetric experiments with N-acetylglucosamine, glucose, and mannose provided enthalpic evidence of distinct binding differences between the three monosaccharides. A comparison of the mode of binding of different monosaccharides is discussed in the context of the antiviral activity of GRFT, based on specific binding to high-mannose-containing complex carbohydrates found on viral envelopes.

Ziolkowska N. E., Shenoy S. R., O'Keefe B. R., McMahon J. B., Palmer K. E., Dwek R. A., Wormald M. R. and Wlodawer A. (2007) Crystallographic, thermodynamic, and molecular modeling studies of the mode of binding of oligosaccharides to the potent antiviral protein griffithsin. *Proteins* **67**, 661-670.

Abstract: The mode of binding of oligosaccharides to griffithsin, an antiviral lectin from the red alga *Griffithsia* sp., was investigated by a combination of X-ray crystallography, isothermal titration calorimetry, and molecular modeling. The structures of complexes of griffithsin with 1 \rightarrow 6 α -mannobiose and with maltose were solved and refined at the resolution of 2.0 and 1.5 Å, respectively. The thermodynamic parameters of binding of 1 \rightarrow 6 α -mannobiose, maltose, and mannose to griffithsin were determined. Binding profiles of 1 \rightarrow 6 α -mannobiose and mannose were similar with K_d values of 83.3 μM and 102 μM , respectively. The binding of maltose to griffithsin was significantly weaker, with a fourfold lower affinity ($K_d = 394$ μM). In all cases the binding at 30 degrees C was entropically

rather than enthalpically driven. On the basis of the experimental crystal structures, as well as on previously determined structures of complexes with monosaccharides, it was possible to create a model of a tridentate complex of griffithsin with Man9GlcNAc2, a high mannose oligosaccharide commonly found on the surface of viral glycoproteins. All shorter oligomannoses could be modeled only as bidentate or monodentate complexes with griffithsin. The ability to mediate tight multivalent and multisite interactions with high-mannose oligosaccharides helps to explain the potent antiviral activity of griffithsin.