1024-Plat Isolating Native Vesicles from Cells and Patterning Them on Fabricated Silane Surfaces

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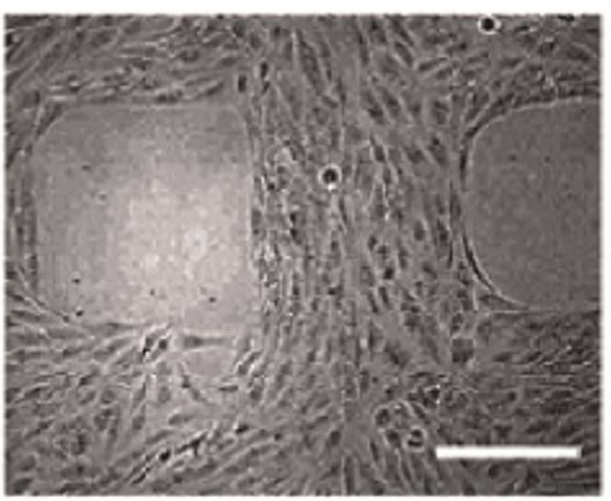
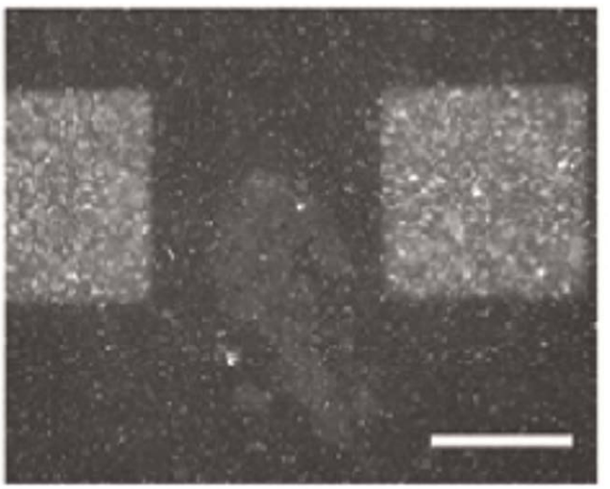
Vesicularlized fragments of plasma membranes (native vesicles) are easier to study, manipulate and store without losing the microstructural integrity and functions of important signaling proteins. Therefore, in lieu of complex whole cells, they may be used as convenient bio-analytical reagents to investigate cellular signaling processes. Here, we describe a one-step and less-invasive technique to isolate native vesicles from human retinal pigment epithelium (RPE) cells by extrusion though polycarbonate filters. Light scattering and fluorescence measurements demonstrate that these post-extrusion cell fragments form closed-shaped spherical vesicles. Fluorescence co-localization studies and single-vesicle Raman spectroscopy establish that they are enriched in glycosphingolipids and cholesterol, typically associated with lipid rafts. Furthermore, they can be deposited into spatial patterns when exposed to amphiphilic surfaces such as patterned n-octadecylsiloxane (OTS) monolayers. Surprisingly, unlike whole cells which avoid highly hydrophobic OTS surfaces, native vesicles display a higher affinity for hydrophobic surfaces, possibly because of their raft-enriched surface chemical compositions. We anticipate that these micropatterned vesicle arrays will open new routes in fabrication of membrane biochips containing functional proteolipidic, supramolecular structures derived directly from cells and possible interesting parallel invesigations of native/synthetic membrane and protein dynamics.





Protein-Ligand Interactions - I

1025-Pos Effect of Force Transducer Stiffness on Unbinding Kinetics Inferred from Molecular Force Spectroscopy

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Board B1

Forced unbinding of complementary macromolecules such as ligand-receptor pairs can reveal energetic and kinetic details governing the mechanisms of extracellular load-transfer and cell-substrate adhesion, as well as physiological processes such as drug metabolism. Molecular-level experiments as well as atomic-level simulations have allowed access to individual dissociation events, and yet even with this detailed information, disparities in measured unbinding force lead to marked variation in inferred binding energetics and kinetics at equilibrium. We investigated these disparities through examination of atomic-level unbinding trajectories generated by steered molecular dynamics simulations as well as through molecular force spectroscopy experiments on the biotin-streptavidin system. We have identified one reason for the experimental discrepancies discussed above. In mathematical analysis of forced ligand-receptor unbinding, the force applied to the ligand-receptor complex is always considered; however, the effect of the force transducer on the energy landscape of the complex has been overlooked. Here, we demonstrate through simulation and experiment that the stiffness of the force transducer can have an appreciable effect on measured ligand-receptor unbinding force. To extrapolate equilibrium kinetic information from forced unbinding experiments, therefore, a range of loading rates as well as force transducer stiffnesses must be considered.

1026-Pos Mapping The Signal Peptide Binding Site In Escherichia coli SecA Using Fluorescence Resonance Energy Transfer

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Board B2

Identification of the signal peptide-binding site within SecA ATPase is critical for understanding the chemo-mechanical cycle of the SecA nanomotor during protein translocation. While recent studies have addressed this topic, the precise signal-peptide binding site on SecA remains controversial. The aim of the present study was to identify the SecA signal peptide-binding site using Fluorescence Resonance Energy Transfer (FRET). FRET provides a more global view of the binding site and circumvents the common limitations of more genetic approaches where deletion and substitution mutagenesis can confound the correct interpretation of protein structure-function analysis. This study employs a collection of functional, monocysteine SecA mutants labeled with a donor fluorophore along with cysteine-containing, acceptor fluorophore-carrying PhoA signal peptides. The equilibrium binding constant of the labeled PhoA SP22Cys or SP2Cys peptides for SecA, as measured by fluorescence anisotropy, was found to be 1.4 uM or 7.8 uM, respectively, while binding stoichiometry was determined to be one signal peptide bound per dimer SecA. Our FRET data with PhoA SP22Cys suggests that the signal peptide-binding site resides within the 221–233 region of the preprotein-binding domain of SecA, consistent with the genetic and biochemical study by Papanikou et al. (2004). We are currently acquiring FRET data with PhoA SP2Cys to confirm these results and also determine the signal peptide orientation on SecA. Additionally, we are measuring the equilibrium binding constant of the labeled PhoA SP22Cys peptide for SecA Delta11, a monomer-bias mutant protein, to determine if there is a signal peptide-binding preference for SecA monomer or dimer.

1027-Pos Pulsed EPR Study of Interspin Distances in MTSL-Lipoxygenase with Doxyl Stearates to Locate Substrate Analog Binding Site

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Board B3

Where and how fatty acid substrate analogs dock on lipoxygenases remains unresolved. Here, initial nitroxide-nitroxide distance measurements, by pulsed EPR at 17.4 GHz, address this subject. Distances are between a spin-label covalently attached near a putative substrate cavity entrance of soybean lipoxygenase-1 (LOX1) and stearate inhibitors (1) spin-labeled at opposite ends of the chains, 5- and 16-DSA. The covalent spin-label (MTSL) is on single Cys mutant, T259C-LOX1.

Lipoxygenase paramagnetic iron ion shortens relaxation times. Values of T1 and Tm for 259-MTSL LOX1, 16- and 5-DSA were determined at 5-65K, confirming that iron relaxation is not dominant in this range and suggesting a hydrophobic environment of both probes. T1's of 259C-MTSL at 50–65K were shorter by a factor of 4–8 than in a typical MTSL-labeled protein not containing a fast relaxing ion. The T1's at 60 K of DSA probes were a factor of 2–3 shorter than T1's of 259C-MTSL. At 10 K, both DSA T1's were ∼equal, indicating that the DSA probe spins are not very close to iron. Shortened T1's at 50–65 K have the benefit of improving SNR by a factor of 2, facilitating faster signal averaging. DEER experiments (6–50 K) on 259C-MTSL-LOX1 plus bound 5- or 16-DSA gave no temperature dependence in the signal shape, consistent with the T1 and Tm determinations. Resolved oscillations were not seen in the decays, indicating some range of nitroxide conformations. From preliminary DEER distance data, the 5-DSA-259C-MTSL system shows distances primarily distributed around 24 Å, while there are two components distributed around ∼27 and ∼18 Å for 16-DSA-259C-MTSL. EPR distance measurements between sites on large proteins and spin-labeled inhibitors have wide potential applications.

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1028-Pos A Quantitative SPM Analysis of a Selectin-mediated Adhesion

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Board B4

Cell rolling mediated by selectins plays a key role in inflammation, stem cell homing, and tumor metastasis. Understanding of binding events between P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) thus holds great scientific importance as the naturally occurring process may be exploited in a variety of biomedical applications to treat diseases including leukemia and metastatic cancer. Although the rolling of leukemia cells (HL-60) has been investigated by several groups, analyses of rolling at a molecular level have relied on computational modeling and indirect measurements due to difficulty of visualization and accessibility of P-selectin and PSGL-1 molecules with nano-scale resolution. Here we present an effective imaging technique using scanning probe microscopy (SPM) that enables direct observation of distribution, number, and spacing of individual P-selectin at nano-scale. SPM-based molecular force spectroscopy was employed to measure binding kinetics between P-selectin and PSGL-1. This nano-scale imaging was enabled by magnetically coated SPM probes that are chemically conjugated with P-selectin or PSGL-1, and specific binding events between P-selectin and PSGL-1 were observed on surfaces of HL-60 cells and P-selectin-immobilized substrates. In addition, the orientation of P-selectin molecules on substrates was

chemically controlled, resulting in maximized binding efficiency between HL-60 cells and P-selectin-conjugated substrates. Based on this study that provides molecular-level visualization of binding events and three-dimensional cell morphology/receptor distribution, a mathematical model based on the experimental data was proposed to predict the movement of rolling cells in the bloodstream.

1029-Pos Time-dependent Inhibition Kinetics Of A Bimolecular Association Is Highlighted By A Novel Surface Plasmon Resonance Assay

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Board B5

Most drugs can only illicit a response when they are bound to their respective molecular target. As such, a key factor in drug design is being able to anticipate the residence time that a ligand remains bound to its target. Residence time, in turn, can be directly correlated with the apparent dissociation rate of the complex. There are examples of therapeutic agents that exhibit slow and tight binding to their molecular target. This means that the rate of dissociation decreases as incubation time increases. Because current methods of high-throughput drug screening do not monitor the off-rate as a function of time, many slow, tight binding drug candidates may be overlooked. We have developed an assay for a surface plasmon resonance (SPR) instrument that can be used to identify bimolecular interactions that are characterized by slow and tight binding. This assay indirectly monitors complex dissociation rate as a function of incubation time. To do this we utilized an antibody that binds Japanese Quail Lysozyme (JQL) and Hen Egg Lysozyme (HEL). Although the apparent affinity of JQL in a short term binding assay is lower than that of HEL, it is an effective competitive inhibitor following a long incubation. Results have been compared with those from a novel time-dependent isothermal titration calorimetery assay. These results indicate that ligands with apparently low affinity in short-term assays may, in fact, be good binders after long incubation times with their target. The development of reliable methodology to study time-dependent residency is important to understanding pharmacological profiles of many ligands.

1030-Pos Predicting Inhibitor Binding Affinities: The Safe_p Approach

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Board B6

The single biggest challenge in the '*in silico*' screening of ligands is the development of a binding free energy or scoring function that could predict accurately the actual binding free energies, or at least the binding ranking of a set of compounds of very diverse chemical structure. We have developed a very simple function, referred to as Solvent Accessibility Free Energy of binding predictor (SAFE_p), which we have applied to inhibitor-binding to the HIV-1 PR, the most studied enzyme as measured by the number of inhibitor-enzyme structures that have been determined by both X-ray and NMR protocols. We show that this free energy of binding scoring function is able to reproduce well the binding affinity ranking of a set of HIV-1 PR inhibitors with a wide variety of chemical motifs and with binding constants differing by several orders of magnitude. We further demonstrate the usefulness of this approach by successfully applying it to the affinity ranking of ligands whose complexes with the HIV-1 PR are not know experimentally, one of most stringent tests of the quality of any binding free energy scoring function. Comparison of the observed against calculated binding free energies indicates that the scoring function is able to reproduce the binding ranking of most of the inhibitors, even in the case that no parameter fitting to the observed affinities has been performed.

Acknowledgments: This work was supported by grants from MEC (Ministerio de Educación y Ciencia, Spain) and Xunta de Galicia. We thank J. L. Martin for sending us the coordinate files of some macrocyclic inhibitors

1031-Pos Experimental and Computational Docking Study of Alcohol Dehydrogenase Stabilization by α-CyD

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Board B7

Alpha cyclodextrin (α-CyD) is a non-reducing oligosaccharide built up from six glucopyranose units. α-CyD is used as an artificial chaperone for the proper refolding of enzymes. Herein, the performed experimental studies by dynamic light scattering (DLS), UV-Vis, circular dichroism spectrophotometry and enzymatic activity assay exhibited that α-CyD resulted in a decrease in the aggregation and an increase in the stabilization of alcohol dehydrogenase (ADH), both kinetically and structurally. Moreover, the resulting data revealed that α-CyD could likely bind the enzyme to the hydrophobic residues from its hydrophobic cavity. For a better analysis of the α-CyD effects on the aggregation decline, computational docking methods were carried out. Molecular docking is widely used to discover new ligands for biological targets with a known 3D structure.

After 4 ns of molecular dynamics simulations performed with the aid of the GROMACS simulation package, the product was docked with the Autodock 3 software at all the ADH regions, based on the blind docking (BD) approach. BD was employed to search the entire surface of proteins for finding the favorable binding regions. The results, deriving from the docking parameters examination of the α-CyD/ADH system, were evaluated with reference to the binding free

energy (ΔG), the population of the Rank and the root mean square deviation (RMSD). Structural analyses disclosed the accessibility importance to the hydrophobic and aromatic amino acid residues, especially located at the dimer's interface. Therefore, it was concluded that CyDs shielded the hydrophobic surfaces of proteins, which were in an aggregation-prone state by its own hydrophobic cavity. Eventually, it could prevent the direct inappropriate interactions between the monomers, causing the protein aggregation.

1032-Pos Ligand Escape Pathways And Free Energy Calculations From Nonequilibrium Simulations: A Computational Study Of The Insulin-phenol Complex

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Board B8

We have applied 'random expulsion molecular dynamics (REMD)' to discover potential escape routes of phenol from hydrophobic cavities in the insulin-phenol complex. Two major pathway classes with several subclasses each have been discovered which provide new insights into (un)binding mechanisms for phenol. We identify several residues directly participating in escape and find agreement with previous NMR results. The exit-point information from REMD trajectories has been utilized to determine reaction coordinates (RC) for dissociation of phenol. These reaction coordinates further serve for choosing pulling directions along various escape routes using steered molecular dynamics (SMD). Additionally, we used the nonequilibrium work theorem (Jarzynski's Equality) with microscopic work information from SMD simulations to construct potential of mean force (PMF) profiles along multiple pathways. The free energy profiles are found to be sensitive to pulling velocity, standard deviation of work values, and initial conditions for finite number of trajectories. Nonetheless, our results for dissociation free energies agree reasonably well with those found previously using relatively much more expensive "slow growth" free energy calculations.

1033-Pos Coupled Protein Conformational Change And Ligand-binding Revealed By Coarse-grained Simulations: Population-shift Vs Induced-fit

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Board B9

Coupling between protein conformational change and ligand binding is a basic property of protein as molecular device. Regarding coupling between conformational change and ligand binding, two extreme mechanisms have been proposed, the population-shift model and the induced-fit model. In the population-shift model, protein is in equilibrium between apo and holo conformations without ligand, then ligand selectively binds to the holo conformation. In the induced-fit model, the ligand weekly binds to apo conformation first, then the ligand interaction induces protein conformational change. Extending a minimal structure-based model, the multiple-basin model, that we developed previously (1) to take into account the ligand interaction, here we simulate coupled dynamics of ligand-binding and conformational change. Varying strength and spatial-range of ligand-protein interaction, we address what condition leads to which of the two mechanisms, finding that the weak and short-ranged interaction gives pre-existing mechanism and *vice versa*.

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1034-Pos Protein-Protein Interactions: Prediction of Binding Sites with the Gaussian Network Model

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Board B10

Residues at the binding sites of the ligand and receptor of a complex are predicted from the slowest and fastest modes of motion by the Gaussian Network Model applied to unbound molecules. The dynamic fluctuations suggest that one of the interacting proteins, mostly the smaller protein referred as ligand, anchor into a structurally constrained region of the other protein, the receptor: The motion of the anchor residue of the ligand is strongly anticorrelated with the rest of the ligand motion in the slowest mode. The motion of the anchoring groove residue of the receptor that is spatially neighboring the anchor residue in the complex structure is strongly correlated with the remaining residues of the receptor in the fastest mode. It is also shown that when the ligand is excited in the slowest mode by a harmonic force, the anchor residue is excited which then couples, through fluctuations, to all other residues of the ligand. Similarly, when the receptor is excited in the fastest mode, the anchoring groove residue which is spatially neighboring the anchor residue in the complex structure is excited which then couples with the remaining residues of the receptor.

1035-Pos Diversity in the Mechanism of Recognition of a Lysine-methylated Peptide by distinct Chromodomains

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Board B11

The post-translational modification of specific lysines along histone H3 tail regulates gene expression through the recruitment of specialized chromatin factors that contain chromodomains. To better understand epigenetic signaling, it is important to delineate the rules that dictate the docking interactions between recognition modules and modified histone tails. Here, we provide evidence that two distinct mechanisms are used by LHP1 and CHD1 chromodomains to recognize methyllysines 27 and 4 in histone H3 tail, respectively. Lysine 27 methylation contributes to gene repression during development, whereas lysine 4 methylation promotes transcription activity. A single chromodomain in LHP1 protein forms a 1:1 interaction with the region of histone H3 tail surrounding methyllysine 27. This methyllysine resides within an ARKS motif which forms complementary interaction with the surface of the chromodomain. The methylammonium group is captured via cation-ϕ bonding by an aromatic cage involving three conserved aromatic residues in LHP1. Interestingly, the ARKS motif also surrounds methyllysine 9 in histone H3 tail. *In vitro* studies support ambivalent binding interaction between LHP1 and methyllysines 9 and 27 in H3 tail. However, *in vivo* characterizations support a dominant presence of LHP1 at chromatin with methyllysine 27. Unlike LHP1, CHD1 protein contains tandem chromodomains that assemble cooperatively to bind a lysine-methylated peptide. The structures of the CHD1 tandem chromodomains in the free and peptide bound states show a substantial difference with LHP1 chromodomain. Our studies provide important insights about methyllysine recognition by single and double chromodomains, and help predict function of uncharacterized chromodomains.

Acknowledgments: We thank NIH for funding and Steven Jacobsen and coworkers for *in vivo* characterization of LHP1.

1036-Pos A Story of Communication Between Remote Regions of an Allosteric Enzyme

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Board B12

Phosphofructokinase from *Lactobacillus delbrueckii* (LbPFK) exhibits weak binding affinity and limited allosteric response to PEP and MgADP when compared to other allosteric PFKs. Sequence and structural alignments between LbPFK and *E.coli* PFK (EcPFK) reveals that the two enzymes are highly similar except for key allosteric site residues. Using site directed mutagenesis, four residues located in the allosteric binding site of LbPFK, H59, E55, D187 and S319, were mutated to their corresponding residues in EcPFK to form HEDS. D12A, a mutation near the active site, was created, as well as K279M, located on the surface of LbPFK distant from either the allosteric or active site. All these mutations were combined to form HEDDS-K, which exhibits enhanced PEP binding when compared to wild-type LbPFK, as well as allosteric inhibition comparable to wild-type EcPFK. Various combinations of these mutations were created to address the role of each residue. E55, D187, K279 and D12 appear to play a role in PEP binding, while S319 appears more important for allosteric inhibition by PEP. EDDS-K displayed enhanced binding affinity for PEP compared to HEDDS-K indicating the lack of participation of H59 in enhancing PEP binding. K279M combination mutants revealed that this mutation has a synergistic effect with the substitution at position 279 limited to methionine and threonine. In the crystal structure, K279 interacts with E277 via a salt-bridge. HEDDS-E277A showed weaker binding of PEP compared to HEDDS-K, indicating that the effect of K279M does not involve breaking of this salt-bridge. Together, these results identify three different regions within LbPFK that are important in enhancing PEP binding and improving allosteric inhibition in LbPFK. Funding came from NIH grant GM33261, the NIH funded CBI training grant and the Welch Foundation.

1037-Pos Not Just A Passive Adaptor, The Periplasmic Component CusB From An RND-type CusCFBA Copper Efflux System Is Chaperoned And Regulated By CusF

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Board B13

CusB is a component of the copper efflux complex CusCFBA of *Escherichia coli.* The Cus system, unlike its multidrug exporter homologs, such as AcrAB-TolC, contains an additional periplasmic component called CusF. CusF is unique to putative copper/silver CBA transport systems and bears no significant sequence similarity to other proteins outside of its orthologs in other organisms. CusC, CusB and CusA, in accordance with CBA-type multidrug exporters, function as the Outer Membrane Factor (OMF), Membrane Fusion Protein (MFP) and Resistance, Nodulation, Cell Division (RND) type antiporter, respectively. Membrane Fusion Proteins are shown to interact with inner and outer membrane components in other CBA type systems. However, their direct involvement in the uptake of periplasmic solutes is not indicated. The periplasmic uptake in Mex and Acr multidrug resistance systems was shown to be mediated by the inner membrane component. In some organisms, the MFP CusB is a single polypeptide with CusF. This suggests an interaction between these two components in Cus system and a possible periplasmic copper uptake by CusB. CusF, which has been shown to bind Cu(I)/Ag(I) through in vivo and in vitro studies might act as a chaperone or a regulator of CusB. In order to understand the molecular details of copper regulation demonstrated by Cus system and more specifically, define the role of MFPs in CBA type metal transporters, we aimed to characterize the MFP CusB and probe its interactions with CusF using various structural and biochemical tools. ITC and NMR were used to determine the nature and specificity of interaction. EXAFS revealed the distribution of metal ion between the two proteins. The study might also enhance our understanding of efflux mediated by CBA type multi-drug exporters.

1038-Pos Key Interactions on Bottom of the Binding Cleft in Neocarzinostatin

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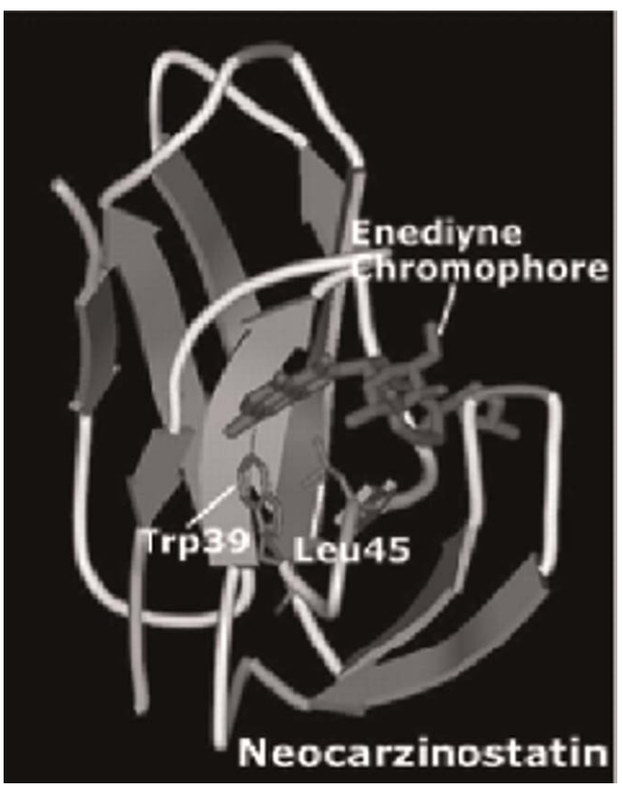
Board B14

Neocarzinostatin is a potent antitumor antibiotic, consisting of a labile enediyne chromophore warhead that is non-covalently bound to a protective carrier protein. We showed earlier that the side chains involved in the opening of the binding cleft can be important in gating the drug release from the holo complex. Here we wish to suggest that the residues at the bottom of the cavity might be crucial for retaining the tightly bound ligand. To evaluate the role of side chains on the ligand binding affinity, site-specific mutagenesis approach was employed at residues located deep in the cavity bottom. Biophysical characterizations by CD and 2D ^{15}N-^{1}H HSQC NMR spectroscopy were analyzed to assure the conformational similarity of mutant proteins to those of wild type. The influence of the mutated proteins on the release rate of enediyne-drug was studied using the reconstituted neocarzinostatin complex. Fluorescence-based kinetic analysis reveals higher drug-release rates from Trp39Ala and Leu45Ala mutant holoproteins than that from wild type. The results were further supported by HPLC analyses on the remaining amount of the protein-bound chromophore. The above observation offers useful insights in the key interactions of the protein-bound enediyne drug.



1039-Pos Mechanism of the Carbohydrate Binding and Conformational Changes in MBP

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Board B15

The binding interactions of the maltodextrins and maltodextrin-binding proteins (MBP) from various organisms have been of great interest. It's because of strong correlations between their specificities and the host-environment interactions and the potential for protein engineering new biosensors. Among these MBPs, *E. coli* MBP is the most studied model which binds to maltodextrins with closing its two domains. However, the binding of the reduced maltodextrins (RM) can be either open or closed configurations. This suggests that the binding of carbohydrates and the closing of two domains are separated events with strong coupling between them. Here, we provide a series of isothermal calorimetry measurements of the interactions between maltodextrins, RM and *E. coli* MBP. Our data show that the binding of RM has a positive heat capacity change (ΔC_p) at temperature below 20 °C and ΔC_p changes to negative above 30 °C. The fluorescence experiments also suggest that RM causes more peak shift of MBP at 37 °C than at 10 °C. Based on these observations, we propose that the binding of RM initially on one domain at low temperature, this binding appears to be driven by hydrophilic interactions such as hydrogen bonds. At higher temperature, the binding involves both domains and energetically coupled with the closing of the two domains. We extrapolate the energy cost of the closing of the two domains is about 7.5 kcal/mol. Furthermore, we used displacement ITC to demonstrate that the constituent glucose moieties' binding is not additive to the final maltose binding.

1040-Pos Structural and Functional Dissection of an Allosteric Protein by Using Amphipathic-Amphoteric Osmolytes

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Board B16

We have studied the allosteric properties of oxygen binding to human hemoglobin (Hb A) in solution, pH 7.4, and in the presence of three kosmotropic osmolytes: betaine, 3-(1-pyridino)-1-propane sulfonate (compound 1), and dimethylbenzylammonium propane sulfonate (compound 2). These compounds present strong amphoteric groups (carboxylate or sulfonate groups, and quaternary amine groups), as well as amphipathic groups.

In the presence of increasing concentrations of betaine (up to 5 M), we observed a progressive decrease in the oxygen affinity for the unliganded form (deoxyHb), whereas no variations were detected for the liganded form (oxyHb). These observations are in accor-

dance with those effects observed in Hb A under osmotic stress conditions.

In the presence of compound 1, a progressive increase in the affinity for the first oxygen was observed with increasing concentrations of the osmolyte (up to 3 M). However, cooperative ligation of oxygen expressed as Hill coefficients, decreased to values slightly lower than 2, indicating that cooperativity was never abolished.

In the presence of compound 2, which exhibits the strongest hydrophobic moiety, oxygenation of Hb showed very distinct characteristics. The increase in oxygen affinity for the unliganded form of Hb in the presence of compound 2 was comparable to that exerted by compound 1. However, in contrast, the affinity for the liganded form decreased significantly, and as a result, cooperativity was severely impaired.

Size exclusion chromatography experiments confirmed that in the presence of compound 1, Hb A tends to dimerize strongly even in the deoxy form. This finding hints at the notion that cooperative binding of oxygen is present even in the dimeric form of Hb A, and that by increasing the hydrophobicity of the osmolyte in the solution, the oxygenation characteristics of Hb A can be altered dramatically.

1041-Pos *RPD31* Encodes Class I Histone Deacetylase Mediates Filamentous Growth In *Candida albicans*

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Board B17

Candida albicans, a fungal pathogen undergoes a morphological transition between yeasts and filamentous growth in response to a variety of external signals. In a previous study, we identified the global repressor Ssn6p acts as an activator as well as a repressor for this filamentous growth. To search the proteins interact with Ssn6p, we investigated the Ssn6p complex after tandem affinity purification and Matrix-assisted laser desorption ionization (MALDI-TOF). From mass data, it is found that the complex includes class I histone deacetylase (HDAC) Rpd31p and global repressor Tup1p. The interaction between Ssn6p and Tup1p, Ssn6p and Rpd31p is confirmed by western blot analysis. To study the interaction between Ssn6p and Rpd31p in detail, we integrated TAP tag into C-terminus of *SSN6* in wild-type and *tup1* mutant cell respectively. As a result, this interaction doesn't need tup1p because Ssn6p-Rpd31p complex was still present in *tup1* mutant cell. To explore the role of *RPD31* in *C. albicans* morphogenesis, we deleted *Rpd31* by homologous recombination using PCR-based long flanking homology region. The *rpd31* mutant was failed to develop hyphae under some of hypha-inducing condition. To investigate the effect of Rpd31 deletion on hyphal specific gene expression, we performed northern blot analysis using some of hypha-specific genes as probe. The expression patterns of hypha-specific genes were consistent with the morphological phenotype. When *RPD31* was overexpressed under hypha-inducing condition, filaments was emerged faster than wild-type strain. From these results, it is suggested that Rpd31 mediates signals for filamentation positively in *C. albicans.*

1042-Pos ITC Studies Of The Binding Of The χ Subunit Of DNA Polymerase III Holoenzyme And The PriA Helicase To *E. coli* SSB And SSB-ssDNA Complexes

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Board B18

E.coli SSB (single stranded binding) protein plays a central role in DNA metabolism, not only because of its essential interactions with ssDNA, but also because it binds and regulates the activity of a variety of other proteins involved in DNA metabolism. The C-terminal unstructured tails of the 4 subunits of the SSB tetramer bind to these proteins and bring them to regions of DNA where they function. We have examined the interactions of SSB with the *Chi* subunit of DNA pol III and with the PriA helicase focusing on their specificity as well as on how these interactions are influenced by the presence of ssDNA. We find that both PriA and *Chi* bind to SSB with stoichiometries of approximately 4 proteins per SSB tetramer, at low (0.02M NaCl) and high (0.2M NaCl) salt concentrations. In low salt conditions (0.02M NaCl) the PriA interaction with SSB is characterized by an association equilibrium constant $K_{obs}=(3–6)\times 10^6\ M^{-1}$ and negative enthalpy change ($\Delta H= -10$ to -15 kcal/mol), that are higher than for *Chi* ($K_{obs}=(3–6)\times 10^5\ M^{-1}$ and $\Delta H=-5$ kcal/mol, respectively). These results indicate that SSB does display specificity towards binding the PriA. These values are in agreement with the binding parameters obtained for a peptide, corresponding to the last 9 amino acids of the SSB C-terminal. To test the effect of DNA binding we investigated SSB binding to the same proteins when SSB is prebound to $(dT)_{70}$ to form a fully wrapped 1:1 complex. We observed that ssDNA increased both K_{obs} and ΔH approximately 10 and 2 fold, respectively, indicating that SSB is more effective recruiting accessory proteins when bound to ssDNA.

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1043-Pos NMR Studies of Methanobactin

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Board B19

Methanobactin (mb) is a small copper binding molecule produced by methanotrophic bacteria. Methanobactin can be isolated from spent media and is also found associated with the copper and iron containing particulate methane monooxygenase (pMMO). A variety of activities are ascribed to mb, including scavenging copper from the environment, serving as a copper chaperone to pMMO, serving as an oxygen radical scavenger, mediating electron flow to pMMO,

and mediating the genetic expression of pMMO. Methanobactin contains seven amino acids along with two unique residues, each containing a thiocarbonyl and an imidazolate group. Methanobactin isolated under low copper levels binds copper(II) with high affinity and reduces it to copper(I). The crystal structure for mb exposed to high copper levels (>1000 Cu:mb) shows one Cu bound per mb and ligated by the two thiocarbonyl sulfur atoms and one of the two nitrogens from each imidazolate group. In the present study we report using NMR to elucidate the structural behavior of mb at low copper levels. We have used a combination of COSY, TOCSY, ROESY and ^{15}N-HSQC experiments to obtain the proton assignments for both the copper bound and uncomplexed forms of mb. We have followed the titration of mb with Cu^{2+} and see marked changes in the ^{1}H spectra. The changes appear complete at around 0.5 Cu:mb and mirror the behavior seen with other spectroscopic methods. These results suggest mb forms a stable homodimer at low copper levels that shares a single copper ion. We have also looked at mb that has had the copper removed after exposure to high levels of copper. We observe a structure similar to the homodimer copper-bound form that suggests the changes occurring upon initial copper binding are irreversible and largely independent of the continued presence of copper.

1044-Pos Determination of Binding Constants of NNRTIs to HIV-1 Reverse Transcriptase

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Board B20

HIV-1 reverse transcriptase (RT) converts single-stranded viral RNA into double-stranded proviral DNA. This enzyme possesses both DNA polymerase and RNase H activities. The biologically active form of RT is a heterodimer composed of a 66 kD (p66) subunit and a 51 kD (p51) subunit; p51 has the same N-terminal polymerase domain as p66 but lacks the C-terminal RNase H domain. In solution, the enzyme exists as an equilibrium mixture of the heterodimer p66/p51, two homodimers p66/p66 and p51/p51, and the two monomers. The homodimers retain DNA polymerase activity and bind nonnucleoside reverse transcriptase inhibitors (NNRTIs). Binding of the inhibitor efavirenz enhances dimerization of all three dimers. The complex equilibria and the effects of NNRTIs on dimerization make it difficult to study the inhibitor binding of RT. The dimerization equilibria are established slowly in the presence and absence of inhibitor. Through a combination of equilibrium dialysis and isothermal titration calorimetry, we are developing a strategy to determine the binding constants of individual NNRTIs to RT dimers and monomers. Determination of the binding constants will provide insight to the thermodynamic linkage of NNRTI binding and dimerization and an important tool for drug development.

1045-Pos Identification of Determinants in Net1 Required for Regulation of the Cdc14 Phosphatase and Coordination of Exit from Mitosis

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Board B21

Cdc14 is a dual specificity phosphatase that is involved in coordinating chromosome segregation and is required for exit from mitosis in *Saccharomyces cerevisiae.* The activity of yeast Cdc14 is tightly regulated in a cell-cycle dependent manner through an interaction with a multifunctional protein known as Net1. Net1 acts as a potent competitive inhibitor by sequestering Cdc14 in the nucleolus and suppressing phosphatase activity from G1 phase until early anaphase. In early anaphase the interaction is disrupted and active phosphatase disperses throughout the cell to act on protein substrates. The precise molecular mechanism by which Net1 inhibits Cdc14 has not yet been defined. To gain insight into the mechanism of Cdc14 inhibition we employed site-directed and truncation mutagenesis to identify determinants within Net1 that are required for binding and inhibiting the phosphatase. These Net1 mutants were analyzed for their ability to bind Cdc14 and to inhibit phosphatase activity. The ability of these Net1 mutants to interact with Cdc14 *in vivo* is also being analyzed. These analyses indicate that Net1 residues 240 to 331 comprise a Cdc14-binding domain. Interestingly, certain truncation mutants failed to inhibit but retained the capacity to bind Cdc14, suggesting there are two distinct sites within the Cdc14-binding domain, one that plays a major role in binding but is dispensable for inhibition and another that contains the elements crucial for inhibition.

1046-Pos Spectroscopic Investigation Of The Molecular Recognition Mechanism Of Methionine Sulfoxide Reductase A

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Board B22

Reactive oxygen species (ROS), often simply by-products of normal cellular metabolism or chemical species inherent to our aerobic environment, attack proteins, often rendering them dysfunctional. The amino acid methionine is particularly sensitive to damage by ROS. While most oxygen damage is irreversible, the enzyme methionine sulfoxide reductase A (MsrA) is capable of repairing methionines in a wide range of damaged substrates. The mechanism of repair for proteins damaged by the oxidation of methionine has been established, but less is known about the initial MsrA-substrate recognition mechanism. We studied MsrA of *E. coli*, a small (23 kDa) monomeric protein that uses a thiol-disulfide exchange mechanism to reduce methionine sulfoxides and requires a reducing agent to regenerate the active site. We hypothesize that MsrA functions as

a molecular chaperone, recognizing overall characteristics of damaged and unfolded proteins. Using intrinsic emission fluorescence spectroscopy, we examined the kinetics of substrate recognition by MsrA and active site regeneration. Previous studies on a peptide substrate revealed multiple-phase kinetics with time constants ranging from seconds to minutes. In the current study we determine recognition kinetics back to the millisecond time scale using stopped-flow methods. To understand how MsrA is able to repair a wide range of target macromolecules, we studied the interactions between MsrA and oxidized and reduced forms of four substrates: staphylococcus nuclease (SN), natively unfolded SN-T62P, a 9-amino acid peptide derived from SN, and the anti-inflammatory drug Sulindac. We determined the extent of repair of oxidized substrates and the secondary structure of the MsrA-substrate complex using complementary techniques of MALDI-TOF mass spectrometry and circular dichroism spectroscopy.

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1047-Pos Why Are Hyperactive Ice-binding-proteins So Active?

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Board B23

Ice binding proteins (IBPs), also called 'antifreeze proteins' or 'ice structuring proteins', are a class of proteins that protect organisms from freezing injury. These proteins have many applications in medicine and agriculture, and as a platform for future biotechnology applications. One of the interesting questions in this field focuses on the hyperactivity of some IBPs (1). Ice binding proteins can be classified in two groups: moderate ones that can depress the freezing point up to ∼1.0 degrees Celsius and hyperactive ones that can depress the freezing point several-fold further even at lower concentrations (2). It has been suggested that the hyperactivity of IBPs stem from the fact that they block growth out of specific ice surfaces, more specifically the basal planes of ice (2). Here we show experimental results based on fluorescence microscopy, highlighting the differences between moderate IBPs and hyperactive IBPs. These include direct evidence for basal plane affinity of hyperactive IBPs, the effects of IBPs on growth-melt behavior of ice (3) and the dynamics of their interaction with ice (4).

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1048-Pos Calorimetric Investigation of a Beetle Antifreeze Protein and Enhancers

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Board B24

Antifreeze proteins (AFPs) were first discovered in the blood of Antarctic fish and have been found in many organisms, such as fish, insect, and plant. They are also known as thermal hysteresis proteins as they can depress the freezing point of the solution by binding to ice and inhibiting its growth without appreciably altering the melting point. Thermal hysteresis (TH) activities are thereby produced. Beetle AFPs are usually more active than fish AFPs and the TH activities of beetle AFPs can be strongly affected by the presence of other substances. The hyperactive properties of the fire-colored beetle AFPs from *Dendroides Canadensis* (DAFPs), in fact, result from the presence of enhancers. Enhancers can be low molecular weight chemicals and macromolecules and some of them do play a role in enhancing the antifreeze activity of AFPs physiologically. Citrate is the best enhancer that has been identified so far for DAFPs. Sodium chloride has only a minor enhancing effect. Differential scanning calorimetry (DSC) has been utilized as a reliable method to study AFPs. We employed DSC to investigate the TH activities of a purified DAFP and the TH activities of the DAFP in the presence of sodium citrate, sodium chloride, and some novel low molecular weight chemicals (as potential enhancers), respectively. The results of the TH activity enhancement of the chemicals are compared and are correlated to the differing physicochemical properties of the chemical. Our results suggest that the efficiency of the low molecular weight enhancers of DAFPs depends on their physiochemical properties, which may affect the binding of the DAFP to ice.

1049-Pos Thermodynamic Studies Of BNP And ProBNP Binding To Anti-BNP mAbs

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Board B25

Brain Natriuretic Peptide (BNP) is an important cardiovascular hormone that consists of 32 amino acids with a disulfide bond formed between residue 10 and 26. It is a valuable diagnostic marker for cardiac disease. A high affinity anti-BNP monoclonal antibody 106.3 has been used to recognize BNP. The epitopic region of BNP was identified using alanine scan and fluorescence correlation spectroscopy (FCS), and later confirmed by X-Ray crystal structure and 2-D NMR. We studied BNP (5–13, peptide containing the epitope region only), full length cyclic BNP (1–32) and pro-BNP

(precursor of BNP which has additional 76 residues at the N-terminus). The dissociation constants and binding kinetics of anti-BNP mAb and the three forms of BNP derivatives were determined by FCS and fluorescence quenching. Comparisons of the thermodynamics results on the three forms of BNP provide insights on how the size and conformation of the antigen affect the antigen-antibody interactions.

1050-Pos Adiabatic Compressibility Variation During Amphiphile Binding To Bovine Serum Albumin

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Board B26

Serum albumin, the most abundant transport protein in the circulatory system, is made of three structurally homologous, predominantly alpha-helical domains. These domains denominated I, II and III, share the two primary protein binding sites existing for a wide variety of ligands/drugs. Since it has been crystallized, the protein has therefore attracted a renewed interest for the understanding of its drug transport and delivery mechanism. We present here the ultrasonic characterization of bovine serum albumin (BSA) during the binding of amphiphile molecules: a cationic surfactant cetylmethyl ammonium chloride (CTAC) and a fatty acid myristic acid (myr). We have investigated BSA by densimetric studies and adiabatic compressibility determinations and monitored the resulting conformational changes by circular dichroîsm. We have investigated first the BSA adiabatic compressibility as a function of CTAC concentration. The compressibility increases up to a CTAC/protein molar ratio of 20, decreasing then for higher ratio values. This result has been interpreted in terms of surfactant binding, water release, protein unfolding and/or aggregation. At the same time the alpha-helical content of BSA was decreasing starting at a ratio around 20. In contrast, volumetric measurements of myristic acid binding reveal a maximum at a myr/BSA molar ratio of 7, which corresponds precisely to the maximum number of binding sites as reported by recent NMR studies. At higher ratio values, a very slow decrease of the compressibility is observed, which might be interpreted by the formation of myr micelles. The binding does not induce observable changes in BSA secondary structure. These results are of value for the design and the developpement of new, tailor-made drug carriers, an important area for future research.

1051-Pos Mutations In PTEN's Phosphatase Domain Affect Binding To Phosphatidylinositol-4,5-bisphosphate and Phosphatidylserine

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Board B27

PTEN is a phosphatase specific for the 3 position of the phosophoinositide ring that is deleted or mutated in many different disease states. PTEN association with membranes requires the interaction of its C2 domain with phosphatidylserine (PS) and most importantly, the interaction of its N-terminal end with phosphatidylinositol-4,5-bisphophate ($PI(4,5)P_2$). We have shown that the latter interaction is specific for $PI(4,5)P_2$ over all other phosphoinositides and this binding specificity is maintained for a short peptide representing PTEN's first 21 amino acids ($PTEN_{1–21}$). We hypothesize that some disease relevant mutations affect the extent and/or specificity of PTEN's binding to $PI(4,5)P_2$ or PS. We have shown that one particular cancer relevant mutation, K13E, leads to a loss of PTEN binding to $PI(4,5)P_2$, suggesting that the lysine in the 13 position is critical for binding. We have investigated several mutants that either vary the nature of the amino acid in the 13 position or alter the position of the lysine by switching it with neighboring amino acids. All mutations encountered abrogate binding of $PTEN_{1–21}$ to $PI(4,5)P_2$ and abolish specificity. Importantly, many of these mutations maintain the overall charge of the peptide, highlighting that the interaction between PTEN and phosphoinositides is specific and not governed only by non-specific electrostatic interactions. $PTEN_{H93R}$ is an autism relevant mutation in PTEN's phosphatase domain associated with a complete loss of *in vivo* activity. Our results indicate that this mutant protein binds more strongly to PS than the wild type protein and that the protein undergoes different conformational changes upon interactions with lipids than the wild type protein. We hypothesize that a new PS binding site is created by this mutation and that the strong binding prevents PTEN from "hopping", which limits PTEN's substrate access and reduces its activity.

Protein-Ligand Interactions - II

1052-Pos Carbohydrate recognition by anti-viral proteins

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Board B28

The surfaces of many enveloped viruses are heavily glycosylated to avoid an immune response; the HIV envelope glycoprotein gp120 is a perfect example. This creates many challenges to the effective inhibition of viral-cell recogition, but also provides unique opportunities. Over the past several years, a number of prokaryotic proteins have been shown to inhibit HIV cell-entry by binding to gp120; these proteins specifically recognize the carbohydrate portion of the glycoprotein. Despite a number of structural and biochemical studies, a complete understanding of the origins of affinity and specificity for diverse carbohydrate targets has yet to be developed.

Using a range of computational approaches, we have developed detailed models of carbohydrate binding to the best-studied of these proteins, Cynanovirin-N. The known differences in binding affinities of a series of oligosaccharides are well-matched by calculations based on these models, and can be explained in simple structural