and it forms hydrogen bonds with highly conserved residues across this interface. Mutating D12 to alanine in BsPFK enhances PEP binding by 100-fold with no change in the extent of PEP inhibition. When D12A was introduced into LbPFK only a 5-fold enhancement in PEP binding was observed. Crystal structures of D12A BsPFK and D12A LbPFK were solved to 2.4 Å resolution. Comparison of D12A and wild-type BsPFK with fructose 6-phosphate bound shows a quaternary shift along the active site interface, breaking the hydrogen bonds involving D12. By contrast, D12A LbPFK exhibits no major change in structure relative to wild type BsPFK. In hopes of further enhancing PEP binding, the following mutations of non-conserved residues in the allosteric site were made to the corresponding residues in either EcPFK or BsPFK, respectively. H59D, E55Y, D187E and S319Y combined showed no enhancement in PEP binding. S211R, D214K and G216S alone and in combination also had no effect on PEP binding. All these mutations suggest that the diminished PEP binding affinity to LbPFK is the consequence of more that just the residues in the allosteric site, likely involving the resistance of this enzyme to undergoing the quaternary shift. Funding came from NIH grant GM33216, NIH CBI training grant, and the Welch Foundation grant A1543.

2289-Pos Board B259

Kinetics and Thermodynamics of the Interaction of ANS with Proteins

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1-anilino-naphtalene-8-sulfonate (ANS) is a fluorescent probe widely used in protein folding and conformational transitions studies. The fluorescent features of ANS, a blue shift of the emission maximum and the increase of quantum yield and lifetime, are generally attributed to the binding at hydrophobic sites. Despite the interaction of ANS with proteins has been extensively studied since the early works of Gregorio Weber, few high-resolution structures of proteins complexed with ANS have been resolved. In this work the binding of ANS to BSA was analyzed at equilibrium and pre-equilibrium conditions. The combined analysis of fluorescence, near UV circular dichroism and isothermal titration calorimetric data provided a detailed description of the binding mechanism. Three ANS molecules bound to BSA in 100 mM phosphate pH 7 at 25°C. Pre-equilibrium experiments allowed to determine the affinity and the relative quantum yield at each binding site by fitting a microscopic model to the fluorescence time-course data. This analysis unambiguously indicated that the binding of ANS to BSA occurs at two different and independent binding sites with similar quantum yields and affinities (ΔG° @ -35 kJ/mol). The binding of ANS to the first site is thermodynamically favored by similar contributions of the enthalpic (ΔH = -16.3 kJ/mol) and entropic terms ($-T\Delta S$ = -19.4 kJ/mol), while the binding to the second site is enthalpically driven (ΔH = -36.6 kJ/mol; $-T\Delta S$ = 4.3 kJ/mol). Complementary information from molecular docking showed 3 ANS molecules bound at hydrophobic cavities in BSA subdomains IIA and IIIA with binding affinities in the order of those found experimentally. The sulfonate group of ANS was oriented towards clusters of polar residues, a common feature in the reported crystal structures of other ANS-protein complexes.
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2290-Pos Board B260

Investigation of the Ligand-binding Mechanism of Methionine Sulfoxide Reductase A of *E.coli*

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The rise of free oxygen in the atmosphere over 2.5 billion years ago made it possible for large land-based plants and animals to thrive. But oxygen, and the energy it provides, comes at great cost. Aerobic metabolism generates highly reactive intermediates and by-products in the form of hydroxyl radicals, superoxide anions and hydrogen peroxide. Within the cell, these reactive oxygen species attack biological macromolecules, producing covalent modifications that can affect both function and structure. One amino acid residue in proteins that is particularly sensitive to oxidation is the sulfur-containing side chain of methionine. Fortunately, methionine oxidation can be reversed by the actions of peptide methionine sulfoxide reductase (MsrA), which reduces methionine sulfoxide back to methionine and restores function to damaged proteins. We have used multiple spectroscopic techniques to investigate the mechanism by which MsrA recognizes and binds to a wide range of oxidized substrates in need of repair. Substrates studied include proteins, peptides and the non-steroidal anti-inflammatory drug Sulindac. Competition experiments with the fluorescent reporter ANS suggest the existence of weak, but specific, hydrophobic interactions between MsrA and unstructured and/or hydrophobic ligands.

2291-Pos Board B261

Characterization of and Kinetics studies on Lipid Extraction of GM2AP Tryptophan Mutants using Intrinsic Fluorescence and a Dansyl-Based Fluorescence Assay

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GM2AP is an accessory protein that functions as a co-factor in degradation of the GM2 ganglioside to GM3. This non-enzymatic lipid transfer protein solubilizes GM2 from intralysosomal vesicles for reaction with HexA. The precise molecular interactions and method of extraction of the GM2 ganglioside from the lipid membrane are not yet known. GM2AP contains four disulfide bonds and three tryptophan residues (W5, W63, W131) with two of these (W63, W131) located in putative membrane binding loops. In this report, the intrinsic tryptophan fluorescence of a series of single and double TRP mutants (W5A, W5AW63A and W5AW131A) of GM2AP is used to characterize protein in solution and in the presence of lipid vesicles. Additionally, results from quenching experiements are shown, where the fractional accessibility of each tryptophan is determined for both neutral and acidic solutions. The kinetics of lipid transfer of each of the tryptophan mutants were also assayed for their ability to extract and transfer dansyl-labeled lipids from liposomes. Removal of the TRP moieties from the putative membrane binding loops results in slower lipid extraction rates, implying that these residues are important in the membrane binding of GM2AP.

2292-Pos Board B262

Binding of Organochloride and Pyrethroid Pesticides To Estrogen Receptors α and β: A Fluorescence Polarization Assay

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Several agricultural pesticides and industrial chemicals, such as the organochlorides DDT and methoxychlor, have been shown to cause both endocrine disruption in humans and binding to the estrogen receptor. Estrogen receptor-α (ERα) and -β (ERβ) are ligand-activated nuclear signaling proteins with widespread presence in the body. Binding of the hormone estradiol to the ER can affect development with an activation profile that is subtype specific. This investigation measures the ability of pesticides to bind *in vitro* to human ERα and ERβ by observing their ability to displace a fluorescent estrogen homologue from the receptor. Eight pesticide related compounds were assayed: four in the DDT family and four based on the natural insecticide pyrethrin. The organochlorides tested were: DDT, HPTE, and two DDT metabolites: VF77-1and VF72-1. The four synthetic pyrethroids tested were permethrin, deltamethrin, bifenthrin and fenvalerate. While all of the molecules in the DDT family that we tested showed strong binding to ERα, the pyrethroids showed either extremely weak (fenvalerate) or no binding (permethrin, deltamethrin, bifenthrin) to ERα under our experimental conditions. ERβ exhibited a different binding profile: high affinity binding to the DDT family of molecules AND to permethrin, lower affinity but still strong binding to deltamethrin and fenvalerate, and no binding at all to bifenthrin. These results suggest that permethrin, in addition to the DDT based molecules, could potentially have the ability to disrupt the estrogen hormone pathway through binding to ERβ. Permethrin's binding to ERβ is notable, particularly in light of its widespread use in home pet care products such as pet shampoo and flea and tick repellants. The results also suggest that the binding affinity of ERβ is similar to but less discriminating than that of ERα.

2293-Pos Board B263

Characterization of the Ca^{2+} Binding Affinity and Coordination Site of the LIN-12/Notch-Repeat

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Notch receptors are transmembrane glycoproteins of a highly conserved signaling pathway that regulate cell growth, differentiation, and death in multicellular organisms. Notch activation requires two successive ligand-induced proteolytic cleavages that enable the intracellular Notch to translocate to the nucleus and regulate gene transcription. Notch proteins exhibit a highly conserved modular architecture, which includes three tandem LIN-12/Notch-Repeats (LNRs) responsible for maintaining the receptor in its resting conformation prior to ligand binding. These highly conserved modules contain a characteristic arrangement of three disulfide bonds and a group of aspartate/asparagine residues that coordinate a Ca^{2+} ion, essential for the correct folding of an LNR. Outside of the Notch family of proteins, LNR modules also exist in proteins such as the PAPP-A and the stealth proteins. In our previous work, we had recombinantly expressed, purified, and refolded the first repeat of human Notch1 and used it as a model system to characterize the binding specificity and affinity of different

metals to an LNR via isothermal titration calorimetry (ITC). In this work, we used a combination of computer modeling and experimental approaches to characterize and compare the Ca^{2+} binding affinities and coordination geometries of various LNR sequences from different proteins. We expect this work to elucidate the basis for Ca^{2+} ion selectivity by the LNRs that is integral for their structural integrity and is required for the proper regulation of the Notch signaling pathway.

2294-Pos Board B264

Regulation of Nuclear PLCβ1 by a Novel Binding Partner called TRAX

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Previous research has led to the discovery that the plasma membrane signaling protein PLCβ1 is sometimes present in the nucleus. Little is known how PLCβ1 is regulated in the nucleus on the plasma membrane. PLCβ1 activity is regulated by G proteins but these have not been found in the nucleus. The focus of this study is to find binding partners for nuclear PLCβ1 and investigate their role in the regulation of its activity in the nucleus. A protein called translin-associated factor-X, TRAX, has been identified as a potential binding partner for nuclear PLCβ1. The work done in this report shows that the two proteins bind in vitro and in living cells. Using a combination of biophysical and biochemical methods, we find the two proteins interact and that TRAX may regulate nuclear PLCβ1 activity.

2295-Pos Board B265

Hybrid Scoring and Classification Using Shape-Based Approaches to Predict Human PXR Activators

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The human pregnane X receptor (PXR) is a transcriptional regulator of many genes involved in xenobiotic metabolism and excretion. Human PXR activators include an extensive range of structurally diverse endogenous molecules, drugs which potentially result in potential drug-drug interactions. Reliable prediction of molecules interacting with this receptor would be valuable for pharmaceutical drug discovery and environmental applications. In the current study, computational models for human PXR activators and PXR non-activators were developed using support vector machine (SVM) algorithms using Shape Signatures and MOE descriptors. The models were validated using separate test sets. The overall test set prediction accuracy for PXR activators with SVM was 72 to 81 % in line with a previous study using VolSurf descriptors and SVM. We have also used the rigorous docking program GOLD and coupled the GoldScore with other scoring functions in an attempt to improve docking results from those previously attained. In this study, the best docking prediction accuracy (61 %) was obtained using 1D Shape Signature descriptors as a weighting factor to the GoldScore. We have also combined the available human PXR data sets into a single larger model (~300 molecules) and described the specific molecular descriptors that we demonstrate can help predict whether a molecule activates PXR. These combined computational approaches could enable us to more confidently identify PXR activators and to further avoid them in various applications.

2296-Pos Board B266

Fluorescence Quenching and Fluorescence Resonance Energy Transfer Studies in the Recombinant N-domain from the Plasma Membrane H(+)-ATPase, Pma1

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Upon substrate binding the isolated plasma membrane H(+)-ATPase (Pma1) from *Kluyveromyces lactis* displays large changes in fluorescence intensity (Sampedro *et al*, 2007 Biochemistry 46:5616-5622). The nucleotide binding domain (N-domain) contains one Trp505 residue, that seems to be responsible for the variations in intrinsic fluorescence. The N-domain was cloned and the protein expressed in *E. coli*. The purified N-domain displayed nucleotide-dependent (ATP and ADP) quenching of fluorescence similar to that observed in the whole Pma1. The dissociation constants (Kd) for ATP and ADP were 100 and 110 uM respectively. Fluorescence resonance energy transfer (FRET) studies were also performed by using mantATP; a fluorescent ATP analog (Ex. 337 nm, Em. 423nm). The absorbance spectra of mantATP overlaps the fluorescence spectra of the N-domain, and thus FRET was observed by exciting at 280 nm. FRET efficiency was 100% indicating a close proximity between Trp505 and the nucleotide. Therefore, in this domain there is a Trp residue located near the substrate binding site which is of high value to determine Kds and molecular distances using fluorescence.

2297-Pos Board B267

NMR Study of the Interaction of Cardiotoxic Drugs with the Extracellular Segment Ile^{583} - Tyr^{597} from the hERG Channel

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Long QT syndrome (LQTS) is a cardiac muscle abnormality caused either by congenital or drug-induced malfunctioning of potassium channels localized in the myocardium cell membranes. LQTS can lead to ventricular arrhythmia and sudden cardiac death. A number of prescription medications inducing long QT have been withdrawn from the market over the past decades, and virtually all cases of drug-induced LQTS are due to the blockade of the heart *human ether-a-go-go-related-gene* (hERG) potassium channel. Evidences show that most of the hERG-channel blockers would exert their activity by binding one or several sites located in the pore region composed of the last two TM helices (S5 and S6) or on the extracellular region connecting S5 and S6 together. In this work, we studied the binding of 4 cardiotoxic drugs (bepridil, cetirizine, diphenhydramine, pentamidine) with a portion of the extracellular segment (Ile^{583} - Tyr^{597}) of the hERG channel and a model membrane. Drug-peptide interactions were studied using ^{1}H liquid-state NMR with pulsed field gradient self-diffusion measurements. According to our CD and ^{1}H NMR results, the peptide appears to be unstructured both in water and membrane mimetic isotropic bicelles. Diffusion measurements suggest that there is no or only weak drug binding to the peptide. However, a strong interaction with the model membrane was evidenced for the bepridil molecule, thus suggesting a potential role of the membrane in the cardiotoxicity of LQTS-active drugs. Our current work, which focuses on drug-membrane interactions and hERG peptide-membrane interactions by ^{31}P and ^{2}H solid-state NMR will also be presented.

2298-Pos Board B268

Protein Selectivity Factors as a Molecular Basis for Metal Toxicity

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Toxic metals are known to displace physiologically-relevant metal ions in proteins, and may activate or deactivate protein function in response to changes in the free metal concentration. To investigate potential relationships between metal/protein complexes and toxicity, an analysis of proteins structural and sequential data was used to establish statistical bases for identifying key selectivity factors associated with Pb^{2+}-protein binding. These data led us to hypothesize that Pb^{2+}, and potentially other toxic metals, may induce opportunistic binding in regions of negative electrostatic potential, thus altering the proteins conformation.

To compare structural/conformational changes, investigate selectivity and affinity, and probe the mechanism of toxic metal-protein interactions, several natural and engineered Ca^{2+}-binding proteins (CaBPs) were analyzed using Fluorescence, CD and 1D and 2D NMR spectroscopy. Engineered proteins were developed based on grafting methods that involved insertion of metal-binding motifs in flexible regions of protein scaffolds to investigate biophysical properties associated with binding reactions in isolated sites. Additionally, the ubiquitous signaling protein calmodulin (CaM) was evaluated extensively to determine changes associated with competitive binding between Ca^{2+} and anthropogenically available toxicants such as Pb^{2+}, Gd^{3+}, La^{3+}, Tb^{3+} and In^{3+}. Results suggest that certain toxic metals may not only displace the biologically-relevant metals in metalloproteins, but support our hypothesis that opportunistic binding occurs in non-sites. This has important implications for the potential binding of toxic metals by non-metalloproteins, as well as providing a basis for understanding the impact of toxicity related to downstream protein-protein interactions.

2299-Pos Board B269

Identification Of The NHERF2 Binding Site For The Chloride/Proton Transporter ClC-5

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The chloride/proton transporter ClC-5 mediates the re-absorption of filtered proteins in the kidney by promoting the formation of the macromolecular endocytic complex and by aiding in endosomal acidification upon complex internalization in proximal tubule cells. Mutations disrupting ClC-5 lead to proteinuria reflecting a severe impairment of renal receptor endocytosis and