

when studying protein binding of small-molecule ligands taken from compound libraries dissolved in organic solvents, as is usually the case during screening or drug development.

Here we introduce a new ITC competition assay that overcomes this limitation, thus allowing for a precise thermodynamic description of high- and low-affinity protein-ligand interactions involving poorly water-soluble compounds. We discuss the theoretical background of the approach and demonstrate some practical applications using examples of both high-affinity ($K_D < 1$ nM) and low-affinity ($K_D > 100$ μ M) protein-ligand interactions.

[1] Velázquez Campoy and Freire, *Biophys. Chem.* **2005**, *115*, 115.

[2] Sigurskjold, *Anal. Biochem.* **2000**, *277*, 260.

1253-Pos

Binding of A Natural Sterol to the Osh4 Protein of Yeast and Membrane Attachment

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Osh4 is an oxysterol binding protein homologue found in yeast that is essential for the intracellular transport of sterols and for cell life. It has been proposed that Osh4 acts as a lipid transport protein, capable of carrying sterols from the endoplasmic reticulum to the plasma membrane (PM).

Molecular dynamics (MD) simulations were used to analyze the binding of ergosterol to the Osh4 protein on an atomic level. During the course of 25-ns simulations, the sterol molecule remained tightly bound to the binding pocket of Osh4. These simulations revealed ergosterol binding was aided by both water-mediated interactions between the 3-hydroxyl (3-OH) group of ergosterol and surrounding polar residues as well as direct hydrogen binding between ergosterol and the Trp⁴⁶ and Gln⁹⁶ residues. Analysis of the interaction energy between ergosterol and Gln⁹⁶ shows distinctly different states (9.3 kcal/mol, 7.3 kcal/mol, and 4 kcal/mol), where the highest energy state was encountered 77% of the time.

In order to study how the Osh4 protein attaches to the PM, possible lipid binding sites were investigated through the use of a docking program as well as MD simulations. A model compound consisting of a phosphocholine lipid head group truncated at the C2 carbon solvated the Osh4 protein in conjunction with water. Protein/lipid interactions were observed and used to determine the proper orientation and placement of the protein with respect to a model membrane. We also developed a model yeast membrane containing ergosterol using CHARMM-GUI, and key membrane properties were investigated using data collected from a 60-ns MD simulation (areas per lipid, density profiles, and lipid dynamics). Ultimately, understanding how Osh4 attaches to the PM will lead to a clear understanding on how this protein transports sterols *in vivo*.

1254-Pos

Splitting of the Allosteric Function in Human Hemoglobin with an Altered Alpha1Beta1 Interface

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Previously, it was shown that human semihemoglobins, i.e., hemoglobin dimers of the form (alpha)(beta) in which only one subunit, either alpha or beta, contained a functional heme group while the complementary apo subunit lacked heme, were sensitive to allosteric effectors, which caused modulation of their affinity for oxygen (Tsuneshige, A. et al. (2004) *J. Biol. Chem.* **279**, 48959-48967). The presented evidences contradicted the classic tenet of the "two-state" model of allostery in which modulation of the affinity for oxygen could only be achieved by a tetrameric hemoglobin adopting the high-affinity "R" or the low-affinity "T" conformations in a reversible fashion.

For the present study, we have prepared a hemoglobin molecule in which the residues alpha104Cys and beta112Cys were chemically modified following a reaction of the sulhydryl groups with a thiopyridyl reagent. These two residues are present in the alpha1beta1 interface, thus we expected that their chemical modifications would impair drastically the intradimeric communication. Surprisingly, oxygenation curves at different pH values, or in the presence of the allosteric effector inositol hexakisphosphate (IHP) showed striking common characteristics: symmetric shape, presence of cooperative binding of oxygen, and a corresponding decrease in overall oxygen affinity in response to acidic conditions or the presence of IHP. Moreover, affinities for oxygen at low and high saturation levels were both affected in similar fashion under any solution condition. These results strongly suggest that the modified hemoglobin behave like dimers exhibiting allosteric properties.

1255-Pos

Contrasting Effects of Halides on the Structure and Function of A Multimeric Allosteric Protein

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We have used two halide salts, namely, sodium chloride and sodium iodide, and studied their impact on the oxygenation characteristics of adult human hemoglobin (Hb). Previous studies in our group showed that both the halide salts exerted similar effects on the Hb function at concentrations below 0.1 M, i.e., an overall decrease in the affinity for oxygen, as a result of a decrease in the affinity at low oxygenation levels. However, as the halide concentrations increased, while chloride continued producing a progressive but rather diminished effect, iodide reverted its effects on Hb: the overall affinity for oxygen rather increased. Careful analysis of the oxygenation curves revealed that while the affinity for oxygen decreased at high oxygen saturation levels, the affinity at low oxygen concentration increased markedly. These effects reached a plateau at a concentration of 2 M, but even more surprisingly, cooperativity was never canceled. The results hinted at the possibility that iodide ions were splitting the tetrameric Hb molecules into asymmetric dimers. Dimers have been and still are considered non-cooperative, high oxygen-affinity systems. Yet, our present data clearly contrast with the previous tenet since cooperativity index showed values as high as 1.6 in the presence of 2 M NaI. Determination of molecular weight by size exclusion chromatography, and the study of oxygenation characteristics of symmetric nickel-iron Hb hybrids in the presence of sodium iodide showed that in fact the tetrameric Hb splits into two dimers that, strikingly, remain allosterically functional.

1256-Pos

Exploring the Conformational Space for the Interactions of Aromatic Residue Analogs with Biologically Important Saccharides

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Proteins interacting with carbohydrate ligands are getting a great deal of attention because of its important role in various biological processes. The crystal structures of several lectin-saccharide complexes have shown the presence of an aromatic residue in the binding site. The C-H hydrophobic patch of saccharide "stacks" against pi-cloud of aromatic residues forming CH-pi interactions, which are governed by dispersive and charge transfer interactions. The energetics of saccharide - aromatic residue interactions are dictated by their mutual position-orientations. It is conceivable that there exist low-energy position-orientations other than those found in the limited number of crystal structures of protein-carbohydrate complexes known to date. Hence, we have explored the conformational space for the interactions of 3-methylindole (3-MeIn), *p*-hydroxytoluene (p-OHTol) and toluene (Tol) (analogs of tryptophan, tyrosine and phenylalanine, respectively) with six saccharides. A Monte Carlo conformational search method was used to explore the features of the molecular potential energy surfaces. We found that the saccharides are densely populated above and below the pi-cloud of the aromatic ring of the amino acid residue but not along the edges. Clustering of conformers indicate the aromatic residues are spread out when interacting with C-H atoms as compared to that with -OH groups. The saccharides were capable of sliding on the surface of the aromatic residue. Four C-H groups can simultaneously participate in CH-pi interaction in 3-MeIn systems owing to its larger surface area. The β -D-Galactose and β -L-Fucose have been found to interact only through their b- and a-faces, respectively. Our ability to understand molecular flexibility through conformational search will further lead to advances in the design of drugs and to understand the advantages of selective choice of aromatic residues viz., tryptophan, tyrosine or phenylalanine in different carbohydrate binding proteins.

1257-Pos

From SPRI (Surface Plasmon Resonance Imaging) Affinity Capture Analysis Up to On-Chip MALDI-MS/MS Analyte Identification

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Multiplex format SPRI analysis allows direct visualization and thermodynamic analysis of biomolecular interactions, and is advantageously used for ligand-fishing of captured bio-molecules on multiple immobilized receptors. Mass spectrometry is a powerful tool for structural characterization and identification. Therefore, the combination of SPRI and MS into one concerted procedure is of a great interest for functional and structural analysis in the fields of proteomics, drug-discovery or diagnostic. We have implemented an on-chip MALDI analysis in which affinity captured bio-molecules are directly detected from the SPR-sensor surface.

The model presented was based on antigens-antibodies interactions. Antibodies, Anti-Beta-lactoglobulin, Anti-ovalbumin and a reference, were arrayed on biochip functionalized with a patented SAM-NHS surface chemistry and the SPR experiments were performed using a MS buffer. A mixture of label free antigens, Beta-lactoglobulin and ovalbumin, was injected and analyte capture was followed in real time. Following the collection of kinetic constants (Kon and Koff) information, the biochip was removed and each spot was submitted

to in situ digestion treatment and MALDI matrix deposition. The SPRi sensor surface was inserted directly in an appropriate MALDI plate holder and MALDI-MS and MS/MS complete identification of the retained antigens was performed directly from each individual spots on the biochip. Using this process, the transfer of the biochip into the MALDI apparatus consecutive to a SPR imaging experiment was straightforward without intermediate treatment that could lead to sample loss and/or contaminations.

A same surface array is dedicated to SPRi for affinity separation and subsequent MALDI-MS and MS/MS identification of the retained ligands from complex solution.

1258-Pos

Thermodynamic Principles of Metal Binding to Biological Systems

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We study the selective binding of the softer K^+ ion over the harder Na^+ and Mg^{++} ions to the 58 nucleotide ribosomal RNA fragment and the S2 site of the KcsA K^+ channel using all atom MD simulations. The ribosomal RNA is interesting in that it binds the K^+ over the harder Mg^{++} ion. We calculate the free energy of exchanging K^+ with Mg^{++} in the binding site relative to the change in the aqueous solution. In order to explain the selectivity, we find that it is necessary to consider the change in the internal energy of the ion binding site. Interestingly, similar physics is observed in the KcsA S2 site. There we elucidate the role of coordination number fluctuations, ion-site binding energetics, and site-site interaction energetics in determining the selectivity of the S2 site for K^+ : once again, the reduced strain in the site in the presence of K^+ (relative to Na^+) explains selectivity. Thus, despite the uncommon origin and completely different chemical composition of the binding site, in both the cases the binding site is constricted in the presence of the harder ion and causes an increase in the unfavorable electrostatic strain. This strain is what leads to the observed selectivity for the softer K^+ ion.

1259-Pos

Effect of Variants at the 78th Residue on Neocarzinostatin Chromophore Release

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Neocarzinostatin is a potent antitumor antibiotics chromoprotein complex. It consists of a labile enediyne chromophore and a protective carrier protein. Release of the bioactive chromophore is the initial key step in its cytotoxic mode of action. We showed that F78 of the carrier protein plays an important role in gating the release. To further study the structural role of 78th side chain in the release mechanism, we made variants at the 78th residue using a PCR-based *in vitro* mutagenesis method. The identity and purity of the expressed protein variants were confirmed by SDS-PAGE, UV, HPLC, and MS spectroscopy. Oxygenation was applied to promote formation of disulfide bonds when necessary, and the integrity of disulfide linkages was verified by a designed iodoacetamide-based test. Thermal denaturation study and CD spectroscopic analysis showed that stability and backbone conformation of these variants were conserved as compared to the wild-type protein. Two-dimensional ^{15}N - 1H HSQC NMR study suggested that the majority of residues around the binding cleft were not disturbed. Reconstitution of the enediyne chromophore into these protein variants showed efficient binding and the binding structure appeared to be similar to that of the native neocarzinostatin. Once the backbone structural effect was excluded, side chain structural effect on kinetic release was studied by monitoring fluorescence changes on samples containing glutathione and the reconstituted variants. The results showed that, except aromatic side chains, the basic variants increased the release rate more significantly than the acidic ones. Depended upon the length, steric hindrance, or hydrophobicity, the variants with aliphatic side chain also increased the release rate at different extent.

1260-Pos

Mechanical Regulation of a Cell Binding Site: Bacterial Adhesins can Distinguish Different Physical States of Fibronectin

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Using extracellular matrix (ECM) fibers, we provide an experimental proof that mechanical forces can disrupt a bacterial binding site. Several bacterial species (gram positive and spirochetes, e.g. *S. Aureus* and *B. Burgdorferi*) express membrane-anchored fibronectin (Fn) binding proteins composed of multiple Fn-binding repeats (FnBRs), each of which bridges several Fn type I modules (FnI). As demonstrated here, the repetitive design of bacterial adhesins enables them to recognize the physical state of a multimodular protein: stretching fibrillar fibronectin causes a structural mismatch switching this multivalent binding site to low affinity as revealed at atomic resolution by steered molecular dynamics and confirmed utilizing a FRET-based Fn fiber stretching assay. The ap-

plication of external mechanical force on the termini of Fn leads to an increase of the intermodular distance between the adjacent FnI modules, and the peptide can no longer bind via multivalent interactions. Even phylogenetically distinct bacteria use similar motifs to bind fibronectin, but have different numbers of FnBRs.

1261-Pos

Quantifying Water-Mediated Protein-Ligand Interactions in a Glutamate Receptor

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It is becoming increasingly clear that careful treatment of water molecules in ligand-protein interactions is required in many cases if the correct binding pose is to be identified in molecular docking. Water can form complex bridging networks and can play a critical role in dictating the binding mode of ligands. A particularly striking example of this can be found in the ionotropic glutamate receptors. Despite possessing similar chemical moieties, crystal structures of glutamate and alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) in complex with the ligand-binding core of the GluA2 ionotropic glutamate receptor revealed, contrary to all expectation, two distinct modes of binding. The difference appears to be related to the position of water molecules within the binding pocket. However, it is unclear exactly what governs the preference for water molecules to occupy a particular site in any one binding mode. In this work we use density functional theory (DFT) calculations to investigate the interaction energies and polarization effects of the various components of the binding pocket. Our results show i) that the ligand and its binding mode dictate the interaction energy of a key water molecule which can be thought of as part of the ligand rather than part of the protein ii) that polarization effects can be large and iii) that the interaction energy of a neighbouring water is particularly large (compared to the other waters in the binding pocket) and may offer a route to compounds with improved affinity if it can be displaced. We discuss the results within the broader context of drug-design.

1262-Pos

Epitope Mapping of Anti-DCP Antibody Using Fluorescence Correlation Spectroscopy

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Des-gamma-carboxy prothrombin (DCP), also known as protein induced by vitamin K antagonist (PIVKA-II), is a recognized clinical marker for hepatocellular carcinoma. Prothrombin contains 10 glutamic acid (Glu) residues within its N-terminus (GLA domain) which are post modified to gamma-carboxyglutamic acid (GLA). DCP is an abnormal form of prothrombin in which some of the 10 glutamic acid residues remain unmodified. A monoclonal antibody was developed to specifically recognize DCP, but not prothrombin. In this study we identified the epitope of the anti-DCP antibody using a series of short peptides representing the GLA domain. For each peptide, a single Glu residue was replaced with a GLA residue. The critical Glu residues recognized by the antibody were identified in a competitive format using fluorescence correlation spectroscopy (FCS). We also evaluated the DCP specificity of the antibody using homologues peptide from various GLA domain proteins present in various blood coagulation factors. The dissociation constant of the antibody was determined using FRET based method.

1263-Pos

The Effect of Alpha-Helix Linker Length on Volatile General Anesthetic Binding to the Four-Alpha-Helix Bundle ($A\alpha_2$ -L38M) $_2$

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In order to investigate the effects of the length of the linker connecting the alpha-helices in the di-alpha-helical protein $A\alpha_2$ -L38M on the overall structure and on the affinity of anesthetic binding to four-alpha-helix bundle ($A\alpha_2$ -L38M) $_2$, we designed constructs containing six and four glycine residue linkers instead of the original eight, respectively. We used site-directed mutagenesis with primers designed to remove two and four glycine residues, respectively, out of eight residues of the original glycine linker present in the four-alpha-helix bundle ($A\alpha_2$ -L38M) $_2$. Variant proteins were expressed in bacteria and purified to homogeneity using reverse-phase HPLC. Protein identities were verified using mass spectrometry. Our initial studies reveal that the variant four-alpha-helix bundle with a four glycine linker binds halothane with a dissociation constant of 1.00 ± 0.05 mM, as assessed using fluorescence spectroscopy. This represents a 10-fold decreased affinity for the volatile general anesthetic compared to the original ($A\alpha_2$ -L38M) $_2$ design, which featured an eight glycine linker. The results indicate that the length of the glycine linker, and the resulting dynamic behavior of the four-alpha-helix bundle protein, dramatically influence volatile general anesthetic binding affinity.