features that are very different from that of Hb A2 and Hb A, consistent with the altered functional properties.

3322-Pos

The Use of Glassy Films and Sol-Gel Matrices to Probe Nitrite Mediated Reactions of Met Hemoglobin

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There have been growing indications that under certain conditions hemoglobin (Hb) can undergo nitrite mediated reactions that result in the formation of bioactive forms of nitric oxide (NO) capable of reversing vasoconstriction due to NO scavenging. This process is especially relevant for the design of Hb based blood substitutes that typically cause vasoconstriction when administered. In this presented work the use of both trehalose-derived glassy films and silane derived sol-gel matrices are used to isolate both reactive intermediates and key steps in nitrite-mediated reactions of met Hb. The glassy films allow for controlled production NO within the glass and controlled access of the NO into the distal heme pocket of the met nitrite derivative of Hb. The use of the sol-gel allows for trapping either the T or R state forms of Hb and for facile separation of products (e.g. nitrosothiols such as GSNO) from the Hb containing sol-gel phase. The contributions of added NO and small thiol containing molecules (L-cysteine and glutathione) are exposed. The results are consistent with the formation of a relatively stable intermediate capable of forming S-nitrosothiols such as GSNO. The intermediate has properties consistent with one proposed by Gladwin, Kim-Shapiro[1] and coworkers which has the potent nitrosating agent N_2O_3 coordinated to a ferrous heme.

[1.] Basu, S., *et al*, *Nature Chemical Biology*, (2007) vol. 3, p.785.

3323-Pos

General Mechanisms for the Folding and Assembly of Myoglobins and Hemoglobins

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Mammalian myoglobin has served as the archetype globin for understanding the folding properties of single domain globins with the 3 on 3 helical fold. After removal of heme, the resultant apo-Mb shows a loss of structure in the proximal F helix and adjacent loops, and during acid or GdmCl-induced denaturation, apo-Mb populates at least one intermediate. In contrast, unfolding of holo-Mb appears to be a simple two-state process with little protein concentration dependence but the underlying mechanism is much more complex. The lack of protein concentration dependence implies that heme either interacts with the unfolded polypeptide, self-associates, or both. The observed steepness of the unfolding curves for holo met-Mb requires that the affinity of hemin for the intermediate and completely unfolded states must be at least be 1000 fold weaker than that for the native apo-state, and as a result, unfolding of holo met-Mb is governed primarily by the affinity of the folded native apo-state for hemin. The generality of this conclusion for holo-Mb has been tested in several other monomeric hemoglobins, including the miniglobin from *Cerebratulus lacteus* and the thermoglobin from *Aquifex aeolicus*.

Human hemoglobin unfolding is even more complex due to association of the α and β subunits into dimers and tetramers. Removal of hemin leads to formation of an apo-$\alpha_1\beta_1$ dimer and its unfolding appears to involve an intermediate whose stability is dependent on protein concentration. This dependence suggests the formation of a dimer intermediate with partially folded subunits still attached to each other through the $\alpha_1\beta_1$ interface. Folding and assembly of holo-Hb is even more complex because there are significant differences in hemin affinity between the α and β subunits, and between tetramers, dimers and monomers.

3324-Pos

Time Resolved Thermodynamic Studies of Ligand Binding/release to Sol-Gel Encapsulated Horse Heart Myoglobin

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The successful confinement of proteins in solid state materials allows for a multitude of applications in the study of protein dynamics, as well as, advances in biotechnologies. The importance of bulk solvent and restricted space on the conformational dynamics of proteins can be identified by encapsulating the biomolecules (peptides, proteins, enzymezes, etc) in environments where both can be regulated. In addition, the affect of encapsulation on ligand binding and preservation of reactivity once encapsulated are of equal importance. With the use of Photoacoustic calorimetry (PAC) and Photothermal beam deflection (PBD) methods along with picosecond-millisecond transient absorption and fluorescence techniques we are investigating enthalpy, molar volume changes, and kinetics associated with with CO dissociation and rebinding to horse heart myoglobin encapsulated in sol-gels. Preliminary PAC data suggest that the dissociation of CO from HHMb results in four kinetic phases: <7ns, ~125ns, ~260ns, and ~600ns. We will discuss the differences in molar volume and enthalpy changes associated with the conformational dynamics of these events in sol-gels relative to native protein in aqueous solution.

3325-Pos

The Role of the Internal Disulfide Bond in the Conformational Dynamics of Neuroglobin

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Neuroglobin (Ngb) is a member of the globin family expressed mainly in brain tissue of mammals and other vertebrates that plays a role in the neuronal response to hypoxia and ischemia. Human Ngb has two cysteine residues (Cys46 and Cys55) within the CD loop and the D helix of the protein that were shown to form an internal disulfide bond, however these two residues are replaced by Gly in rat Ngb and the internal disulphide bond is missing. Therefore, to investigate the impact of the internal disulfide bond on the dynamics and energetics in Ngb we used photoacoustic calorimetry and transient absorption spectroscopy and determined the time-resolved volume and enthalpy changes associated with CO rebinding to human Ngb, rat Ngb and a rat Ngb mutant with and engineered internal disulfide bond (Cys46rNgb). The relaxation of the protein structure associated with the ligand photo-release is fast (< 50 ns) and involves a decrease in the volume of the protein matrix. The enthalpy change associated to CO photo-dissociation for all samples studied was 19 kcal mol^{-1}, whereas the reaction volume changes for human Ngb and Cys46rNgb were roughly two times higher than for rat Ngb. The reaction volume changes obtained for human and Cys46rNgb were 13.4 ± 0.9 mL mol^{-1} and 10.3 ± 0.6 mL mol^{-1}, respectively, and 4.6 ± 0.3 mL mol^{-1} for rat Ngb. These results indicate that the presence of the engineered internal disulphide bond in Cys46rNgb leads to a structural volume change that is similar to that found for human Ngb indicating that the internal disulphide bond control, to some extend, conformational dynamics associated with the ligand binding to deoxy Ngb.

3326-Pos

Design and Characterization of an Enzymatically Active Amphiphilic Maquette Protein

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Many questions still exist about how quinone molecules act as substrates for membrane oxidoreductase enzymes, as well as how quinones can act as a catalyst in energy conversion mechanisms. We apply our knowledge of electron tunneling and protein design towards defining the basic engineering requirements for quinone reactivity in natural membranes and heme proteins. We have synthesized and characterized a transmembrane, amphiphilic maquette protein, AP6, which extracts the basic structural components from Complex III necessary to perform transmembrane proton-coupled electron transfer. We have shown that our AP6 peptide assembles as a four-helix bundle protein and can potentially bind up to six bis-histidine ligated hemes tightly across a membrane interface. Given its sequence and heme binding capabilities, our AP6 design could accomplish a variety of potential functions, including: transmembrane electron transfer, electron transfer with aqueous proteins, proton-coupled electron transfer, or combining these, quinol-cytochrome *c* oxidoreductase activity. Through standard Complex III activity assays, we have demonstrated that AP6 has quinol-cytochrome *c* oxidoreductase activity in detergent micelles that is within two orders of magnitude of the activity of natural Complex III purified from *R. capsulatus*. This activity can be generated with a variety of reduced quinone substrates, and is dependent on the concentration of cytochrome *c* present. With no obvious quinone-binding site included in our protein design, AP6 provides clear evidence that a specific quinone-binding site within a membrane protein is not essential for generating significant quinol-cytochrome *c* oxidoreductase enzymatic activity from a heme protein.

3327-Pos

The Effect of Non-Coordinated Water in the Heme Pocket on the Ligand Binding Dynamics of Heme Proteins

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Water molecules in internal protein cavities play fundamental roles in satisfying the H-bonding potentials of main chain atoms in turns, coils, and loops,

determining the stability and rigidity of proteins, shifting the pKa values of buried ionizable residues, and modulating dynamical processes such as folding, catalysis, and proton transfers. Detecting these internal water molecules is sometimes obscured in x-ray crystallography due to positional disorder. We have developed a spectrokinetic assay that accurately detects the presence of a non-coordinated water molecule in the distal heme pocket of myoglobin and in a series of distal pocket mutants, including many where this water molecule is positionally disordered. We also have shown that this water plays a major role in determining the observed bimolecular recombination rate constant. We show that 1) this water molecule modulates the ligand binding dynamics of a series of H64, L29 and V68 mutants; 2) it plays the major role in the observed pH dependence of the CO recombination kinetics between pH 4 and 7 with the protonation of the distal histidine acting as a switch to change water occupancy; and 3) it may also modulate ligand binding dynamics in isolated hemoglobin chains, with the occupancy being larger in the alpha chains. Accurately measuring water occupancy in heme proteins answers crucial questions about water in apolar or slightly polar protein cavities and clarifies the role internal water molecules play in modulating protein function.

3328-Pos

Experimental and Computational Study of the Monomer-Dimer Equilibrium in Dehaloperoxidase from Amphitrite Ornata

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The enzyme dehaloperoxidase (DHP) from the annelid Amphitrite ornata is a unique hemoglobin that functions as a peroxidase, capable of converting 2,4,6-trihalophenols (TBP, TCP, and TFP) into dihalogenated quinones and other products. The DHP protein crystallizes as a dimer. Yet, it was originally characterized as a monomer in solution. We have conducted small angle X-ray scattering (SAXS) in order to probe the monomer-dimer equilibrium in solution. The interest in this area arises from the fact that many hemoglobins are multimers that play an essential role in the cooperativity of oxygen uptake and release. For example, A. ornata possesses a giant hemoglobin (erythrocruorin) like many other marine organisms. Since there are only two known hemoglobin genes (DHP A and DHP B) in A. ornata, it is logical that one or both of these proteins associate with other proteins to form the giant hemoglobin. On the other hand, coelomic DHP does not appear to have a high degree of cooperativity. Moreover, the dimer interface in the X-ray crystal structure of both DHP A and DHP B consists of only 3 amino acid residues. The SAXS data show that the equilibrium for DHP favors the monomer form up to the highest concentrations studied (~ 200 micromolar). However, is there a small amount of the dimer in solution. Thus, it is of interest to apply the known interfaces from study of the X-ray crystal structure to determine which surfaces of DHP may be interacting weakly in solution. We studied the monomer-dimer interface using molecular dynamics (MD) simulations in order to ascertain the relative strength of these interfaces. These results are used to develop a systematic approach to characterization of monomer-multimer equilibria based SAXS and X-ray crystallography data.

3329-Pos

Heme Proteins: The Role of Solvent on the Dynamics of Gates and Portals Revealed by MD Simulations

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In the family of respiratory proteins, hemoglobins and myoglobins have been the first to be crystallized in '50. Despite the availability of 3D structures, issues regarding the microscopic functioning remain open, such as, for instance, the R to T switching mechanism in hemoglobin or the ligand escape process in myoglobin. Due to the relatively small number of residues, myoglobin is the suitable candidate to investigate the more general structure-function paradigm, being defined as the hydrogen atom of biology. In this work, to complement our recent study on the dynamics of internal cavities of myoglobin[1], the effect of solvation on these intrinsic pathways has been explored. In particular, 60ns-long molecular dynamics simulation of horse heart met-myoglobin was further analyzed and the dynamics of waters residing around/inside the protein with average residence times of up to tens of nanoseconds was monitored. Together with the knowledge obtained previously[1], the analysis of solvent revealed that myoglobin has in fact only few stable hydration sites in which a water molecule can stay for time longer than 2 ns. Strikingly, all of these sites are close to protein/solvent portals observed in previous studies focused on the entry/escape and migration of various ligands in myoglobin[2-4].

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3330-Pos

The Effect of Distal Heme Pocket Mutations on the Water Accessible Areas in Myoglobin

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Internal water molecules are important to protein structure and function. A non-coordinated water molecule in the distal pocket of a myoglobin has been shown to be the dominate factor in controlling the binding of CO to the heme active site. We previously developed a method to experimentally measure the entry of internal water into the distal pockets of Mb mutants after photodissociation of CO. In order to better understand what factors control the occupancy of this disordered water in the protein we compared the occupancy with the size of the mutated residue and hydrophobicity. We see little correlation between residue size and water occupancy and a good correlation between water occupancy and hydrophobicity. In order to better understand what factors contribute to internal water occupancy, we further examined how cavity volume and the dynamic behavior of the distal histidine influence water occupancy. Using a computational approach, we calculated the internal volumes of myoglobin cavities for various mutants. We further characterized these cavities by investigating the dynamic behavior of the H64 residue using molecular dynamics. The data show high flexibility of the H64 in the wild type protein suggesting a mechanism by which water is allowed access to the distal cavity. However, in the distal pocket mutants, the H64 can adopt a more stable conformation thereby reducing water access to the cavity. These findings suggest that the flexibility of the distal histidine plays a key role in influencing water access to the distal cavity and the binding affinity for gaseous ligands. In addition, the long range molecular dynamics was used to assess stability of the cavity bound water for the various mutants. The obtained data showed correlation between hydrophobicity and the water residence time in the cavity.

3331-Pos

Ferryl Intermediates in Heme-Based Dioxygenases

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In contrast to the wide spectrum of cytochrome P450 monooxygenases, there are only two heme-based dioxygenases in humans, tryptophan dioxygenase (hTDO) and indoleamine 2,3-dioxygenase (hIDO). hTDO and hIDO catalyze the same oxidative ring cleavage reaction of L-tryptophan (L-Trp) to N-formyl kynurenine (NFK), the initial and rate-limiting step of the kynurenine pathway. Despite immense interest, the mechanism by which the two enzymes execute the dioxygenase reaction remains elusive. Here, we report the first experimental evidence for a key ferryl intermediate of hIDO. It supports a new mechanism, in which the two atoms of dioxygen are inserted into the substrate via a consecutive two-step reaction. This finding introduces a paradigm shift in our understanding of the heme-based dioxygenase chemistry, which was previously believed to proceed via simultaneous incorporation of both atoms of dioxygen into the substrate. The ferryl intermediate is not observable during the hTDO reaction, highlighting the structural differences between the two dioxygenases, as well as the importance of stereoelectronic factors in modulating the reactions.

3332-Pos

Modulation of the Conformation of Cytochrome *c* Oxidase from *paracoccus Denitrificans* by Active-Site Mutations

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We have measured the resonance Raman spectra of the wild-type (wt) and 8 different mutants of cytochrome *c* oxidase from *Paracoccus denitrificans* (pdCcO). Most of the mutants bring about large changes in the binuclear center