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

including conversion of heme a_3 from high spin to low spin and/or disruption of the H-bonding environments surrounding the formyl groups of both heme *a* and heme a_3. To obtain a semi-quantitative measure of the conformational changes induced by the mutations, we use CO as a structural probe. In CcO, the Fe-CO moiety typically exhibits two conformations, called the α and β forms. The Fe-CO stretching mode of the α form is present at ~ 520 cm^{-1}, whereas that of the β forms appears at ~490-495 cm^{-1}. The α form, which is the active conformation of the enzyme, has Fe-CO and C-O stretching modes that do not fall on the $\nu_{Fe\text{-}CO}$*vs* $\nu_{C\text{-}O}$ inverse correlation line characteristic of heme coordinated by a histidine ligand, presumably owing to the interaction of the CO with the nearby Cu_B atom in the binuclear center. Our data of the CO-bound pd*Cc*O showed that α/(α+β) intensity ratio varies from nearly zero to one in the mutants. The changes in the α/(α+β) ratio correlate well with changes in some of the heme modes. We postulate that the conformation of the catalytic site, consisting of the two heme groups and Cu_B, is perturbed by the mutations, as indicated by the changes in the heme modes, which disrupts of the juxtaposition between Cu_B and the iron atom of heme a_3 as reflected by the changes in the α/(α+β) ratio. The implications of these results in relation to the measured functional properties of the enzyme will be discussed.

3333-Pos

Characterization of the Radical Intermediates of Dehaloperoxidase A and B from Amphitrite Ornata

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The enzyme intermediates of dehaloperoxidase (DHP) from the marine worm *Amphitrite ornata* are unique within both the globin and cytochrome c peroxidase superfamilies. Both isoenzymes of DHP, termed A and B, have been shown to oxidize trihalophenols to dihaloquinones in a dehalogenation reaction that utilizes hydrogen peroxide as a co-substrate. We have shown that the initially formed heme intermediate in this reaction is not Compound I as is often the case in peroxidases, but rather is a combination of an iron(IV)-oxo (Compound II) and a tyrosyl radical that together have similarity to the Compound ES intermediate of cytochrome c peroxidase. In order to possibly identify the origin of this radical species in DHP, we have expressed the tyrosine mutants DHP A (Y34F), DHP A (Y38F), DHP A (Y34F/Y38F), and DHP B (Y38F), and studied their reaction with hydrogen peroxide using a combination of stopped-flow UV-visible and rapid-freeze quench electron paramagnetic resonance spectroscopies. Although each mutant exhibited an average signal at $g \approx 2.0058$ confirming the presence of a protein radical, significant differences in the lineshape and width of each radical was observed. We have further characterized these mutants using biochemical assays to determine their effect on the catalytic activity of the enzyme, and relate these results to the structure of the heme active site. Such mutagenesis studies of DHP provide critical insight into the mechanistic details of the H_2O_2-dependent oxidative dehalogenation reaction catalyzed by dehaloperoxidase, present a clearer description of the function of DHP at the molecular level, and lead to a better understanding of the paradigms of globin structure-function relationships.

3334-Pos

Investigation of the Low Frequency Dynamics of Heme Proteins: Native and Mutant Cytochromes $P450_{cam}$ and Redox Partner Complexes

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Vibrational coherence spectroscopy investigates the low frequency dynamics of cytochrome $P450_{cam}$ upon binding to its electron transfer partner putidaredoxin (Pd) and its camphor substrate. A strong correlation between the ''detuned'' coherence spectrum and the Raman spectrum is demonstrated. There is a striking appearance of a mode near 103 cm^{-1} in $P450_{cam}$ when camphor is not present in the distal pocket. This reflects a specific heme distortion, such as saddling, in the substrate free state where water is coordinated to the low-spin iron atom. A mode near 78 cm^{-1} intensifies when the $P450_{cam}$/Pd complex is formed, suggesting a ruffling distortion, possibly related to increased electron donation from the thiolate sulfur. The L358P mutant exhibits similar spectroscopic properties to that of wild type $P450_{cam}$ when bound to Pd[1]. The appearance of a mode near 65 cm^{-1} in the coherence spectra of the L358P mutant reveals similarities to the perturbations seen in the $P450_{cam}$/Pd complex, consistent with the view that the heme and its environment in the L358P mutant are similar to the Pd-bound native protein. Resonance Raman spectra are presented for both $P450_{cam}$ and the L358P mutant. When the native and mutant samples are compared, a ~ 5cm^{-1} red-shift of the mode at ~345 cm^{-1} (without substrate) and a ~2cm^{-1} shift (with substrate) are observed. Such changes are due to the thiolate ligand being pushed towards the heme in the mutant sample. Vibrational coherence spectra of the 2Fe-2S cluster proteins ferredoxin (Fd) and putidaredoxin are also presented. Fd displays vibrational overtones of a mode at 44 cm^{-1} mode, along with a weak mode at 283cm^{-1} that is associated with the labile sulfur-iron stretching vibration.

[1] T.Tosha *et al*., *J.Biol.Chem*., **2004**, 279, 42836.

3335-Pos

Block the Inhibitor Binding Site in the Interior of Dehaloperoxidase from Amphitrite Ornata

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Dehaloperoxidase (DHP A) from the annelid *Amphitrite ornata* is a catalytically active hemoglobin-peroxidase that possesses an internal binding site in the distal pocket and an external binding site near the heme edge. We have recently demonstrated that DHP A has a unique two-site competitive binding mechanism, in which the internal and external binding sites communicate through two conformations of the distal histidine (H55). The native substrate is 2,4,6-tribromophenol, but DHP A is capable of oxidizing any 2,4,6-trihalophenol to the corresponding dihaloquinone and other products. While DHP A is very effective at oxidizing 2,4,6-trichlorophenol, assays of DHP A on 2,4-dichlorophenol and 4-chlorophenol show little to no activity. Binding of 4-halophenols in the internal site prevents oxidation of trihalogenated phenols at the external site, i.e. they are inhibitors. X-ray crystallography shows that when para-halogenated phenols (4-iodo-, 4-bromo-, 4-chlorophenol) bind internally, the halogen is accommodated by a hydrophobic cavity that is analogous to the Xenon 4 binding site in Sperm whale myoglobin. Using resonance Raman spectroscopy, we demonstrate that the apparent dissociation constants of the para-halogenated phenols mimic the trend observed in the X-ray crystal structures. The results suggest that a few amino acids (L100, V59, F21, F24, and F35) surrounding the hydrophobic cavity regulate internal binding of the inhibitor. Using site-directed mutagenesis, we have changed several of these amino acids to prevent internal binding, and thus to increase DHP A activity towards mono- and dichlorinated phenols. Mutation to tryptophan (F21W and V59W) gives rise to new radical intermediates which complicates the interpretation in terms of inhibitor binding. Alternatively, aliphatic amino acids and phenylalanine provide steric effects that can alter the oxidation of rates of 4-chloro-, and 2,4-dichlorophenol.

3336-Pos

Cytochrome c Oxidase CuA and Heme A: Redox Equilibrium and Interactions

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Reduction of detergent-solubilized formate-inhibited beef heart cytochrome c oxidase +/- cytochrome c in turnover with ascorbate was followed aerobically. Heme c, heme a and CuA steady states were monitored. Heme a and CuA were in equilibrium with each other, and with cytochrome c when the latter was present. In the formate system there is no aerobic reduction of any binuclear centre component (heme a3 or CuB). At pH 7.4 and 30 deg C calculated E0'values were +310 mV for heme a and +260 mV for CuA, assuming E0'for cyt. c of +255 mV. The difference in heme a and CuA redox potentials permits determination of separate difference spectra for the two. Oxidized (cupric) beef heart CuA has positive absorbancies in the 500-600 nm region in addition to the characteristic 835 nm band, as previously shown for some isolated bacterial oxidase CuA-containing subunit II preparations. The 605nm peak of reduced heme a is red-shifted in presence of oxidized CuA. Both heme a and CuA titrations are close to simple Nernstian one-electron processes, indicating almost no redox interaction between the centres in the formate-inhibited system. Reported difference spectra of bacterial cytochrome c oxidases and separated CuA-containing subunits show similar features in the visible region but marked differences in the NIR bands, with beef heart and Rhodobacter CuA red-shifted compared with Paracoccus and Thermus CuA. The results affect the kinetic analysis of the enzyme as well as the use of the CuA steady state in determining the functional status of the terminal oxidase in vivo.

3337-Pos

Thermodynamic Profiles of Heterotropic Allostery of Hemoglobin (Hb) by Isothermal Titration Calorimetry (ITC)

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The O_2-affinities of deoxy- and oxy-states of Hb ($K_T$and K_R) are reduced up to 60- and 2,000-folds, respectively, by interactions with potent heterotropic allosteric effectors such as BPG, IHP, BZF, and L35 (1,2). These heterotropic allosteries of such magnitudes mean that heterotropic allosteries are the principal regulatory function of Hb rather than the homotropic allostery (cooperativity), since the latter provides an allostery of only ~30-folds (1). It should be noted