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

that such hetrotropic allosteries are accompanied by no static quaternary or tertiary structural changes (3). Even α- and β-semi-Hb, though they are αβ-dimers, exhibit a heterotropic allostery of up to 100-folds (4). Thermodynamic profiles of these heterotropic allosteries of Hb have been measured by ITC, in order to assess the nature of the interactions of Hb with heterotropic allosteric effectors. Supported by an NIH grant, HL14508.
References: (1) Yonetani & Laberge (2008) Biochim. Biophys. Ac; 1784, 1146-1158; (2) Yonetani et al. (2002) J. Biol. Chem. 277, 34508-34520; (3) Yokoyama et al. (2006) J. Mol. Biol. 356, 790-801; (4). Tsuneshige et al (2004) J. Biol. Chem. 279, 48959-48967.

3338-Pos
Role of His(E7) in Regulating Ligand Binding to the Subunits of Human HbA
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To resolve previous discrepancies between structural and functional studies, the role of the distal histidine in HbA was re-evaluated by preparing Gly, Ala, Leu, Gln, Phe and Trp(E7) mutants and measuring the effects on O_2, CO, and NO binding to mutant/wild type hybrid tetramers and isolated mutant subunits. Substituting His(E7) with apolar amino acids dramatically increases O_2 dissociation (20-500-fold) in both subunits, suggesting equally strong hydrogen bonds between His(E7) and bound O_2 ($\Delta G_{H\text{-}bond} \approx -5.6$ kJ/mol). Increasing the size of the E7 residue from Gly to Phe results in monotonic decreases in the bimolecular rates of ligand binding to both subunits, supporting the E7 gate as the pathway for ligand entry in HbA. The results for the Trp(E7) mutants are more complex. Both fast (~150-200 $\mu M^{-1}s^{-1}$) and one or more slow phases (1 to 0.1 $\mu M^{-1}s^{-1}$) are observed after photolysis of CO. The fraction of the fast phase decreases markedly when [CO] is lowered. In contrast, when isolated α and βTrp(E7) deoxyHb subunits are mixed with CO in stopped flow experiments, only slow phases are observed. Thus, after photolysis of the CO form of Trp(E7) mutants, there appears to be a competition between bimolecular ligand rebinding to an ''open'' conformation and the movement of the indole side chain back into the E7 channel forming an equilibrium ''closed,'' slowly reacting conformation. This mechanism is supported by the crystal structure of the CO form of α (wt)/βTrp(E7), in which the mutant indole side chain is in an open conformation exposed to solvent. In the deoxyHb crystal structure of αTrp(E7)/β (wt), the indole ring of Trp(E7) is in a closed conformation, blocking both the ligand binding site and the E7 channel for ligand entry.

3339-Pos
Resonance Raman Spectra of an O2-Binding H-NOX Domain Reveal Heme Relaxation upon Mutation
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Resonance Raman and electronic absorption spectra were measured for the wild type **H**eme-**N**itric oxide/**OX**ygen binding domain from *Thermoanaerobacter tengcongensis* (*Tt* H-NOX WT) and three other *Tt* H-NOX proteins containing mutations at key conserved residues to determine the heme conformation in solution. The most dramatic changes in heme conformation occurred in the O_2-bound forms, and the single *Tt* H-NOX P115A mutation was sufficient to generate a significant relaxation of the chromophore. Clear evidence of heme relaxation in the *Tt* H-NOX I5L, P115A, and I5L/P115A mutants in solution is demonstrated by the observation of reduced resonance Raman intensities for several out-of-plane low frequency modes (e.g., γ_{11}, γ_{12}, γ_{13}, and γ_{15}) in the 400-750 cm^{-1} region known to be sensitive to ruffling and saddling deformations, as well as increased vibrational frequencies for the core heme skeletal stretching modes, ν_3, ν_2, and ν_{10}. In addition, all three mutants exhibited some degree of heme conformational heterogeneity based on several broad skeletal markers (e.g., ν_{10}) in the high frequency region. These results are comparable to those observed by Olea et al. for *Tt* H-NOX P115A in crystal form, where four different heme structures were determined from a single unit cell. On the basis of the resonance Raman spectra, it is clear that the actual heme conformation for *Tt* H-NOX P115A in solution is considerably more relaxed than that of the WT protein, with increased flexibility within the protein pocket, allowing for rapid sampling of alternate conformations.

3340-Pos
Crystal Structures of Proton Uptake Mutants of Cytochrome c Oxidase in Reduced and Oxidized Forms: Loss of Key Waters Account for Inactivation
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Two proton uptake routes, D and K pathways, have been defined in *Rhodobacter sphaeroides* cytochrome c oxidase by structural and mutational analysis. Single mutations in either pathway, D132A or K362M, strongly inhibit activity but do not change the spectral properties. To clarify the structural basis of the inactivation, the mutants were crystallized in reduced and oxidized forms.
The D132A mutation causes a change in conformation at the mouth of the D path, shifting residues 130-135. The D132 carboxyl is replaced by a density significantly greater than water, which is best fit by a chloride ion. The waters and residues in the D-pathway are unchanged, except for a water that is hydrogen-bonded to N207; the water is lost and the side chain of N207 shifted 2 Å. These minor changes appear to be responsible for the major change in enzyme activity (2% wildtype). The reduced crystal (at 2.15 Å resolution) shows a movement of the heme a_3 porphyrin ring similar to that seen in wildtype (Qin et al., Biochemistry 48:5121, 2009), but to a lesser extent. Changes at the heme a_3/Cu_B site also differ from wildtype, suggesting a mixture of two forms.
In the K362M crystal there are no obvious residue movements: the methionine occupies the same position as the lysine. However, the water associated with K362 is missing. When reduced, the K362M crystal shows conformational changes similar to wildtype. The strong inhibition of K362M (0.02% wildtype) appears to be accomplished with only the loss of one key water. (GM26916 (S.F.M.))

3341-Pos
Unraveling the Mystery of Ferricytochrome C: An Investigation into Induced Non-Native Conformational Changes
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Cytochrome_c, in its oxidized state, adopts a multitude of conformations depending on solution conditions. Some of these conformations are relevant for the protein's functions in the electron transport process and in apoptosis. As frequently as cytochrome_c has been investigated limited experiments have been carried out under low ionic conditions, which is of significant biological importance since it's required for Apaf-1 complex formation and anionic lipids. This investigation explores the energy landscape of cytochrome_c under well-defined thermodynamic conditions. A comparison of CD and absorption of the Soret band region of both the native and non-native states of ferricytochrome c adopted between pH1-13, and temperatures between 278-353K at ionic strengths below 0.1mM was performed. Avoiding the binding of anions to positive patches on the proteins surface. State-I shows the protein unfolded with the iron in a high spin state, as the protein environment was acidified a Cotton band emerges in the CD spectra, the intensity of the bands decreased, starting around pH4. Approaching state-III, the iron enters a low spin state, a stronger couplet emerges reflecting band-splitting, predominantly caused by a combination of electronic and vibronic perturbations, maintained below 343K. Suggesting a conformational transition from the native state, into a thermally activated intermediate state, affecting the internal electric field causing moderate rearrangements of the heme, until it enters a thermally unfolded state. This state of the protein consistently becomes populated at higher temperatures across the pH range. This couplet remains into pH9 possible reflecting an intermediate transition of state III-IV, moving more alkaline this couplet disappears. Using Kuhn anisotropy, $\Delta\varepsilon/\epsilon$ vs temperature, the population of intermediates is indicated as temperature increased. Characterization of ferricytochrome_c transitions at low ionic strength showed significant heterogeneity of the protein throughout the pH range.

3342-Pos
Dynamic Control of Ligand Entry into the Heme Cleft of Cytochrome c1 in the bc1 Complex from *Rhodobacter sphaeroides* - A Four-Site Saga
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Binding of small molecules to the heme of soluble *c*-type cytochromeshas provided insight into the protein conformational dynamics that allow exogenous ligand access to the heme cleft and drive the rupture of the methionine-Fe bond. We have probed the heme domain of membrane-bound cytochrome c_1 through the binding of imidazole (Im) to oxidized cyt c_1 of detergent-solubilized bc_1 complex from *Rba. sphaeroides.* Binding of Im to cyt c_1 substantially lowers the heme E_m and fully inhibits bc_1 complex activity. Binding was tight ($K_d \approx 330$ μM) and enthalpically driven. The rate of formation of the cyt c_1-Im complex exhibited several regions of imidazole concentration dependence: upto 3 mM the rate was linear with [Im] but then increased in a parabolic fashion; at [Im] >20 mM the rate leveled off, indicating a rate-limiting conformational step with lifetime ~0.9 s; at [Im] > 100 mM, the rate substantially increased again, also with a parabolic dependence on [Im]. The overall kinetics were well described by binding at four sites, two high affinity ($K_B \approx 110\ M^{-1}$) and two low affinity ($K_B \approx 1\ M^{-1}$), with distinct reaction rates. Imidazole binding and release rate constants exhibited very large activation