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
Ivan Birukou[1], Rachel L. Schweers[1,2], John S. Olson[1].
[1]Rice University, Houston, TX, USA, [2]Katten Muchin Roseman LLP, Chicago, IL, USA.
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
Rosalie Tran, Elizabeth M. Boon, Michael A. Marletta, Richard A. Mathies.
University of California, Berkeley, CA, USA.
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
Jian Liu[1], Ling Qin[2], Carrie Hiser[1], R. Michael Garavito[1], Shelagh Ferguson-Miller[1].
[1]Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI, USA, [2]Sandia National Laboratories, Livermore, CA, USA.
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
Jonathan B. Soffer, Reinhard Schweitzer-Stenner.
Drexel University, Philadelphia, PA, USA.
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
Oleksandr Kokhan, Vladimir P. Shinkarev, Colin A. Wraight.
University of Illinois, Urbana, IL, USA.
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

energies and huge preexponential factors. Neither the complex dependence of the binding rate on [Im] nor the thermodynamic and activation properties of the reaction are consistent with the popular model for *c*-type cytochromes in which ligand binding is preceded by a spontaneous opening of the heme cleft that becomes rate limiting at high concentrations. Instead, the dependence is better explained by initial binding of ligand that facilitates access to the heme by disruption of the H-bonded network of the heme domain.

3343-Pos

Electronic and Geometrical Structures of Hemes *a*/a_3 of the Bovine Cytochrome *c* Oxidase in the Fully-Reduced and Oxidized States Revealed by *ab Inito* Calculations

Mauro Boero[1,2], **Jiyoung Kang[1]**, Masaru Tateno[1,2].
[1]Graduate School of Pure and Applied Sciences, University of Tsukuba, Tsukuba, Japan, [2]Center for Computational Sciences, University of Tsukuba, Tsukuba, Japan.

In the respiration process, cytochrome *c* oxidase (C*c*O) plays a crucial role; i.e., it generates the gradient of the proton concentration between a matrix and an intermembrane space of the mitochondria, exploiting the free energy released by the catalysis where an oxygen atom is reduced to two water molecules. For this reaction, the required electrons/protons are transferred to the heme *a* site and the heme a_3-Cu_B site of C*c*O. Then, the catalytic reaction and proton pumping process are simultaneously occurred through these sites. In this way, the heme systems act as ''hub'', which is essential to understand the functional mechanisms of C*c*O.

In this study, to investigate the electronic structures of the heme systems, the Wannier function centers (WFCs) were employed. The WFCs correspond to the centers of maximally localized orthogonal functions termed the Wannier functions, and the concept of WFCs is similar to the Lewis view of the molecular bonds. Accordingly, this may provide convenient pictures of electronic structures based on chemical intuitions.

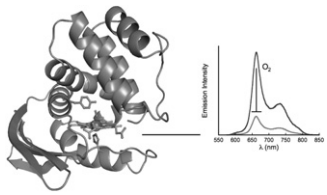
Using the bovine C*c*O structures, we constructed isolated models, each of which contains a heme and a histidine ligand, and performed geometry optimizations using *ab initio* DFT/HF hybrid all-electron calculations at the B3LYP functional level. Then, using the wave functions calculated for each of the heme systems, we obtained Wannier functions and WFCs. As a result, we have found that the imidazole ring of the histidine ligand are rotated in both the reduced and oxidized states, and are strongly associated with the polarization of the heme systems. In C*c*O, the hemes contact with the amino acid residues through van der Waals interactions, which could regulate the electronic structures of the heme systems.

3344-Pos

Examining Oxygen Accessibility in H-NOX Proteins with a Phosphorescent Ruthenium Porphyrin

Michael B. Winter[1], Emily J. McLaurin[2], Steven Y. Reece[1], Charlie Olea Jr.,[1], Daniel G. Nocera[2], Michael A. Marletta[1].
[1]University of California, Berkeley, CA, USA, [2]Massachusetts Institute of Technology, Cambridge, MA, USA.

H-NOX (**H**eme **N**itric oxide and/or **OX**ygen) binding proteins are found in organisms from bacteria to humans and include soluble guanylate cyclase (sGC), the principle mammalian receptor for nitric oxide (NO). The ferrous heme of sGC selectively binds NO - completely excluding oxygen (O_2) binding - and resists oxidation in air. These features allow sGC to respond to NO even in aerobic cellular conditions. In the present study, we investigated the ability of O_2 to access the sGC heme pocket by incorporating a phosphorescent ruthenium porphyrin into two sGC domain truncations to serve as an internal O_2 sensor. A crystal structure obtained for a homologous H-NOX domain suggests that incorporation of the ruthenium porphyrin does not alter the H-NOX fold. In the sGC domains, differences were observed in both emission intensities and lifetimes +/- O_2, providing the first direct evidence that O_2 accesses the sGC heme pocket. In addition, the extent of O_2 quenching was found to parallel oxidation rates in the domains. Together these findings highlight the complexities of O_2 discrimination in sGC and point to the role of higher protein structure in partially protecting the heme from O_2.



3345-Pos

Structural Dynamics in Chloroperoxidase: A Photoacoustic Study

Simona Horsa, Xiaotang Wang, Jaroslava Miksovska.
Florida International University, Miami, FL, USA.

Chloroperoxidase (CPO) is the most versatile heme containing enzyme that exhibits peroxidase, catalase, halogenase and monooxigenase activities. The heme iron coordination resembles to P450s enzymes whereas the composition of the distal pocket is more polar, containing Glu183 hydrogen bounded to His 105 and the heme pocket is connected with the surrounding solvent by two hydrophobic channels. To investigate the ligand-protein interactions in CPO we have employed time-resolved photoacoustic calorimetry and transient absorption study. Unlike other peroxidases, the CO photo-dissociation from CPO can be described as a two step process with distinct volume and enthalpy changes. The photodissociation of Fe-CO bond is associated with negligible volume change ($V = 1 \pm 8$ mL mol^{-1}) and a positive enthalpy change ($H = 48 \pm 34$ kcal mol^{-1}). Subsequent CO escape from the protein matrix is characterized by a positive volume change (25 ± 8 mL mol^{-1}) and insignificant enthalpy change (-2 ± 33 kcal mol^{-1}) and occurs with a lifetime of ~ 20 ns. Similar thermodynamic parameters were found in D_2O at pD 4.6 but the CO photo-release occurs two times slower (~ 50 ns) than in phosphate buffer. Presence of substrates (halides or cyclopentanedione) did not show a significant impact on the thermodynamic profiles associated with the ligand dissociation.

Membrane Protein Structure II

3346-Pos

Charge-Charge Interactions Promote Transmembrane Helix-Helix Association Depending on Sequence Context

Dieter Langosch, Jana Herrmann, Angelika Fuchs, Johanna Panitz, Stephanie Unterreitmeier, Dmitrij Frishman.
TU Muenchen, Freising, Germany.

Folding and oligomerization of integral membrane proteins frequently depend on specific interactions of transmembrane helices. Interacting amino acids of helix-helix interfaces may form complex motifs and exert different types of molecular forces (Herrmann et al., 2009; Langosch and Arkin, 2009; Unterreitmeier et al., 2007). Here, a set of strongly self-interacting transmembrane domains, as isolated from a combinatorial library, was found to contain basic and acidic residues in combination with polar non-ionizable amino acids and C-terminal GxxxG motifs. Mutational analyses of selected sequences and reconstruction of high-affinity interfaces confirmed cooperation of these residues in homotypic interaction. Probing heterotypic interaction indicated the presence of interhelical charge-charge interactions. Further, simple motifs of an ionizable residue and GxxxG are significantly overrepresented in natural transmembrane domains and a specific combination of these motifs exhibits high-affinity heterotypic interaction. We conclude that intramembrane charge-charge interactions depend on sequence context. Moreover, they appear important for homo- and heterotypic interactions of numerous natural transmembrane domains.

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Langosch, D., and I.T. Arkin. 2009. Interaction and Conformational Dynamics of Membrane-Spanning Protein Helices. Protein Sci. 18:1343-1358.
Unterreitmeier, S., A. Fuchs, T. Schäffler, R.G. Heym, D. Frishman, and D. Langosch. 2007. Phenylalanine Promotes Interaction of Transmembrane Domains via GxxxG Motifs. J. Mol. Biol. 374:705-718.

3347-Pos

Revisiting Hydrophobic Mismatch with Free Energy Calculations of Transmembrane Helix Tilting

Taehoon Kim, Wonpil Im.
The University of Kansas, Lawrence, KS, USA.

Membrane proteins are involved in various cellular processes via interactions between their helices and the surrounding lipid environments. It is energetically favorable for transmembrane (TM) domain to match the hydrophobic thickness of the lipid bilayer. We have utilized a helix tilt restraint potential and the free energy decomposition technique to characterize the microscopic forces governing tilting of various TM peptides in different conditions. To investigate the influence of hydrophobic length of TM helix on its tilting, we used a model peptide called WALP (acetyl-GW2(LA)nLW2A-NH2) by varying systematically the length of the peptide (WALP16, WALP19, WALP23, and WALP27). Also, to study the influence of membrane hydrophobic thickness on TM helix tilting, we used two kind of lipid bilayer (DMPC and POPC). Finally, we used arginine, lysine, and alanine as a flanking residue instead of tryptophan (acetyl-GX2(LA)8LX2A-NH2: X = R, K, and A) to examine the influence of various anchoring residues on TM helix tilting. The detail structural features and energetic contribution from hydrophobic (mis)match between lipid and peptides are presented and discussed.