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