for how lipid composition and curvature control fusion kinetics. Here we report simulations where both the composition and the curvature of lipid membranes were varied, with curvature regimes ranging from small 15-nm vesicles to larger vesicles to planar bilayers. We also consider the effects of curvature fluctuations in bilayers. We find a pronounced effect of curvature on fusion rates; in addition, the effects of lipid composition remain relatively independent of curvature over the systems simulated. We then apply these models to interpret the effects of fusion proteins on membrane curvature.

## 1883-Plat SNAP-25: Palmitoylation and membrane interactions

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Understanding neurotransmitter release requires defining the cellular machinery and environmental factors essential for synaptic vesicle exocytosis. We have studied SNARE-induced fusion using a model system based on planar lipid bilayers (BLMs) and native secretory vesicles. In this system, SNAP-25 can function as a v-SNARE and the loss of SNAP-25 from vesicles reduces fusion rates. To further define the role of SNAP-25 in fusion, we examined its interaction with other SNARE proteins and with membranes subsequent to altering SNAP in several ways. Using an environment sensitive tryptophan fluorescence assay, we observe a distinct change in SNAP-25's interaction with membranes following palmitoylation or oxidation of the cysteines in the membrane-associating region of SNAP25's linker region. Palmitoylation was performed in the presence of the palmitoyl transferase HIP14 (huntingtin interacting protein 14). Palmitoylation was confirmed by using a modification of Green's assay, which includes removal of palmitic acid with hydroxylamine followed by biotinylation and detection with streptavidin-HRP (Drisdel and Green, BioTechniques *36*:276–285. 2004). Exact quantification of palmitoylation is difficult because the cysteines are close together and exactly one streptavidin (with four binding sites) can bind multiple biotinylated cysteines. This ambiguity can be reduced by pre-incubating streptavidin with biotin (1:3.5), thus producing a streptavidin with just one binding site. Palmitoylation of SNAP-25 may be important not only for targeting of SNAP-25 to the membrane, but also in allowing SNAP-25 to modify or be modified by the fusing membranes.

## 1884-Plat Effect Of Sphingomyelinase-Mediated Generation of Ceramide on Aggregation of Low Density Lipoproteins

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The Response-To-Retention hypothesis states that retention of aggregated LDL within the intima is the single event necessary and sufficient to provoke atherosclerosis. We became interested in this hypothesis and an observation reported ex vivo that LDL aggregates tend to be nominally 100 nm diameter, or "64-mers." We demonstrated in earlier studies that LDL aggregation follows principles of colloid science and is well-described by a mass action model. In the case of sphingomyelinase(Smase)-induced aggregation, we demonstrated that the relative number of LDL and enzyme molecules, rather than individual concentrations, determines the final aggregate size. While a 100 nm aggregate size can sometimes result in enhanced uptake by macrophages, there does not appear to be anything special about 100 nm per se. In the current study, we extend these ideas and perform a careful measurement of ceramide generation during LDL aggregation. Ceramide is assayed by resorufin fluorescence for magnesium (Mg2+)-dependent Smase in the enzyme concentration range 0.00–0.22 units/mL at a substrate concentration of 0.33 mg LDL/mL. All reaction profiles were fit with a single model involving a unique set of five kinetic rate constants (traditional Michaelis-Menten parameters, plus irreversible binding between ceramide and Smase, an enzyme diffusion time lag, and binding of enzyme to substrate). Identical conditions were used in complementary LDL aggregation studies involving dynamic light scattering (cumulant fits to determine light-scattering-intensity-weighted average diffusion coefficients and effective aggregate diameters via Stokes-Einstein equation). Whereas temporal profiles of both aggregation and ceramide exhibit expected Smase concentration dependencies, a non-obvious result ensues when aggregation is plotted versus ceramide generation; all data fall onto a single, straight line. This reduction of data onto a single master curve reinforces the notion that fundamental principles can and do apply to the study of biologically-relevant phenomena.

### Heme Proteins

## 1885-Pos Effects of Distal Pocket and Subunit Interface Mutations on Ligand Binding in Hemoglobin

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### Board B1

Structural and functional studies have been performed to investigate the effects of αL29F, αL29W, αV96W, βN108K, αV96W/βN108K, αV96W/αL29F/βN108K and αV96W/αL29W/βN108K substitutions on the ligand binding properties of human hemoglobin. The αL29F and αL29W mutations, which are located in the ligand binding pocket, exhibit a slow phase for ligand association after complete and partial photolysis, which reflects a change in the intrinsic ligand-binding properties of the mutant subunit itself. This has been investigated further by examining $^1$H-NMR spectra of partially saturated samples. The βN108K mutation, located in the $\alpha_1\beta_1$ interface also exhibits a slow phase after complete photolysis, but in this case, the amplitude and rate constants suggest the presence of a population of 'T'-state tetramers rather than a change in the intrinsic ligand-binding properties of either subunit. The αV96W substitution is known to introduce a novel hydrogen bond

into the $\alpha_1\beta_2$ interface, and to stabilize the low affinity 'T'-state. Accordingly, rHb(αV96W/βN108K) exhibits larger slow phases after both complete and partial photolysis. These results suggest that:

1. the distal pocket mutations act at the level of intrinsic subunit affinity,
2. the interfacial mutations act at the level of allosteric state, and
3. the effects appear to be additive.

These conclusions are supported by $^1$H-NMR spectra, showing structural perturbations local to the heme pocket in rHb(αL29F) and rHb(αL29W), and more general perturbations for rHb(αV96W) and rHb(βN108K). The multiple mutant HbCO spectra have small peaks in characteristic 'T'-state marker positions.

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## 1886-Pos FTIR Studies Of Cytochrome ba3 From Thermus Thermophilus

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**Board B2**

The ba3-type cytochrome c oxidase from Thermus thermophilus is a member of the heme copper oxidase superfamily. It catalyzes the reduction of molecular oxygen coupled to proton pumping. In order to understand how the proton pump works in cytochrome ba3, it is critical to know the sites of the various protonation/deprotonation reactions during the catalytic cycle. Proton movements inside the protein are achieved by "hopping" along hydrogen bonds involving water molecules, polar residues and ionizable residues. Previous work using time-resolved FTIR spectroscopy showed that an acidic residue is perturbed upon flash photolysis of CO from the reduced enzyme [Biophys.J. 2004,86(4):2438–44]. It was speculated that residue D372, located near a heme a3 propionate, might be responsible for the FTIR signal, and might be an important residue in the proton pump mechanism of the ba3 oxidase. In the current work, attenuated total reflection (ATR)-FTIR difference spectroscopy and site-directed mutagenesis were used to identify acidic groups in the ba3 oxidase from T. thermophilus whose hydrogen bonding or state of protonation is perturbed upon changing from the fully oxidized to the fully reduced state of the enzyme. An FTIR signal from one or more acidic groups is present in the difference spectrum of the wild type enzyme. The D372I mutant oxidase retains the FTIR spectral features, showing that D372 is not responsible for FTIR changes evident in the redox difference spectrum of the wild type oxidase, and is, therefore, unlikely to be responsible for the previously observed changes accompanying CO photolysis. It is more likely that these FTIR signals are due to perturbation of one or more heme propionate groups. In addition, the FTIR difference spectrum has features consistent with perturbation of a histidine residue, which we are currently investigating.

## 1887-Pos Out-of-plane Deformations Of The Heme Group Of Different Ferrocytochrome C Proteins Probed By Resonance Raman Spectroscopy

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**Board B3**

We measured the low-frequency polarized resonance Raman spectra of horse heart, chicken, and yeast(C102T) ferrocytochromes c with Soret excitation. We examined the out-of-plane deformations of the heme groups by determining the relative intensities and depolarization ratios of a variety of Raman bands assignable to out-of-plane (oop) vibrations. Out-of-plane deformations are functionally relevant for different species of heme proteins, in that they affect different properties such as ligand binding affinities, electron-transfer rates and redox potentials. The presence and intensity of bands from $B_{1u}$ modes, $\gamma_{10}$- $\gamma_{12}$, indicate that ruffling ($B_{1u}$) is the dominant heme deformation for all cytochromes investigated, in agreement with results from X-ray structure analysis. The analysis of relative Raman intensities and depolarization ratios of the low-frequency bands shows differences in nonplanarity of the heme group of yeast(C102T) compared to horse heart and chicken cytochromes c. Yeast(C102T) displayed less of a ruffling deformation than horse heart and chicken. Deviation of depolarization ratios of 0.125 from Franck-Condon active Raman modes indicates the presence of other out-of-plane deformations, i.e. doming ($A_{2u}$) and propellering ($A_{1u}$), suggesting yC102T has a larger contribution from these oop deformations. Our results are in full, quantitative agreement with the heme structures derived from the X-ray structures of the respective proteins and disagree with prediction from Molecular Dynamics simulations. This shows that heme deformations derived from crystal structures are representative for the respective solution structure, so that they can be utilized to calibrate Raman intensities of out-of-plane modes for investigating functionally relevant structural changes of the active side.

## 1888-Pos Spectrokinetic Detection of Non-Coordinated Water Entry into the Distal Heme Pocket of Photodissociated MbCO

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**Board B4**

Internal water molecules sometimes play an important role in normal protein function but their presence may be obscured in x-ray crystallography by positional disorder. A functionally important

non-coordinated water molecule in the distal heme pocket of myoglobin slows the ligand binding rate and lowers the observed affinity of ligands to the heme iron. We developed a novel kinetic assay that accurately detects the presence of non-coordinated water in the distal heme pocket of myoglobin. The hydration assay uses a nanosecond time-scale blue shift in the deligated 560-nm absorption peak as a marker for the rapid entry of a water molecule into the distal heme pocket after CO photodissociation. We apply this assay for non-coordinated water entry into the heme pocket of WT myoglobin and a series of distal pocket myoglobin mutants to show that:

1. The kinetic analysis provides a quantitative assessment of non-coordinated water occupancy in the heme pocket, showing that this value is an important factor controlling the observed bimolecular recombination rate constants of many distal pocket mutants studied.
2. The increase in the CO biomolecular recombination rate at pH 4 is due primarily to changes in water occupancy.
3. The slow binding of some mutants, such as L29F, is due to a positionally disordered water molecule in the distal pocket that is detectable using this kinetic assay.

Accurately measuring water occupancy in myoglobin answers crucial questions about water in apolar or slightly polar protein cavities and clarifies the role this water plays in modulating protein function.

## 1889-Pos Roles of Amino Acid Residues in the Distal Heme Pocket on the Structure-Function Relationship in Human Hemoglobin

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**Board B5**

This study explores the structural and functional effects of amino acid substitutions at helical position E11, located 3–6 Å from bound $O_2$ in the distal heme pocket of both α- and β-chains of human normal adult hemoglobin (Hb A). Four recombinant hemoglobins (rHbs), rHb (αV62L), rHb (αV62I), rHb (βV67L), and rHb (βV67I), have been expressed in *Escherichia coli* and purified. $O_2$ affinity, Bohr effect, cooperativity of the oxygenation process, auto-oxidation, and $^1$H-NMR spectra have been measured for each mutant. Substitution of Leu in either subunit, or Ile in the α-subunit does not result in significant functional deviations in the $O_2$ affinity, Bohr effect, and cooperativity from those of Hb A. However, rHb (βV67I) shows a two-fold decrease in $O_2$ affinity while maintaining the cooperativity. As expected, these mutations perturb the ligand-binding site of their respective subunits, as evidenced by $^1$H-NMR. rHb (αV62L) and rHb (αV62I) exhibit a small perturbation of the $\alpha_1\beta_2$ interface in the deoxy state, showing that the quaternary structure of the tetramer is more sensitive to added volume at position E11 in the α-chain than in the β-chain. Auto-oxidation studies have shown that these mutants undergo faster oxidation than Hb A. In comparison to the other three mutants, rHb (βV67I) is most resistant to oxidation. Mutations that generate the highest rates of auto-oxidation (αV62I and βV67L) also produce the greatest structural perturbation in the heme-pocket region. Study of these mutations enhances our understanding of the heme-pocket environment and will improve our understanding of the structure-function relationship of hemoglobin.

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## 1890-Pos Optical Absorption and Resonance Raman Investigations on Novel Pigment P-574

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**Board B6**

Optical absorption and resonance Raman spectra were measured for the novel *E. coli* pigment P-574. The measurements were taken on whole *E. coli* cells (strain RKP2624) using microscopic spectroscopy techniques. We could establish the absolute absorption spectrum of the reduced form of this pigment. The spectrum is similar to those of heme compounds, but visible bands (533, 576 nm) are unusually intense as compared to the Soret band. Furthermore, the Soret band (450–460 nm) is red-shifted from the standard band position of ferrous hemes. The resonance Raman spectra measured for the reduced form indicate a 6-coordinate (low spin) ferrous heme, but the $D_{4h}$ symmetry of porphyrin ring is suggested to be broken. The pigment P-574 was found to be unstable upon oxidation. On the basis of these findings, we propose a possible structure of P-574.

## 1891-Pos Myoglobin-Bound Alky Isocyanides Indicate Diatomic Ligand Pathways

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**Board B7**

Globins reversibly bind oxygen and other diatomic gases to their heme prosthetic group. Their protein superstructure sequesters the heme from solvent to inhibit its oxidation but still provides access to the active site. To investigate the locations of ligand pathways within sperm whale myoglobin (Mb), we have re-examined crystal structures of Mb containing bound n-alkyl isocyanides (CNR) and measured the stretching frequencies and geminate rebinding kinetics of these complexes in solution. These ligands bind to the heme iron through their isocyano group, leaving the alkyl tail to occupy nearby space that offers the least steric resistance and, therefore, may represent possible pathways of escape and entry for the smaller diatomic gases. When n-butyl isocyanide is bound to wild type recombinant Mb in P6 crystals, the alkyl tail points toward the interior of the protein, whereas in native Mb in P21 crystals, it points

toward the closest protein-solvent interface, opening the distal HisE7 "gate." FTIR spectra of both wild-type and native n-butyl isocyanide Mb complexes have two peaks in the isocyano stretching band, indicating that both ligand conformations are present in solution in approximately equal proportions. The FTIR spectra of ethyl and n-propyl isocyanides bound to Mb have a higher percentage of the inward-pointing conformation. Geminate recombination experiments with the corresponding MbCNR complexes demonstrate that outward-pointing isocyanides escape rapidly to solvent, whereas those that point inward rebind rapidly on nanosecond time scales. Together, these structural, spectral, and kinetic data support a model in which dissociated ligands initially move to the interior of heme pocket but then return and exit the protein by way of the distal histidine gate. These results also demonstrate that long chain alkyl isocyanides can be used as "Ariadne's thread" to identify ligand escape routes in globins.

## 1892-Pos On The Molecular Mass Of The Extracellular Hemoglobin Of *Glossoscolex Paulistus*: Analytical Ultracentrifugation Re-examination

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**Board B8**

The giant extracellular hemoglobin of *Glossoscolex paulistus* (HbGp) is constituted by subunits containing heme groups with molecular masses (M) in the range 15–19 kDa, monomers of 16 kDa (***d***) and trimers of 51–52 kDa (***abc***), and non-heme structures, linkers (24–32 kDa). Based on subunits *M* determined by mass spectrometry and HbGp stoichiometry, $12(abcd)_3L_3$, the predicted *M* for HbGp oligomer is 3,410 kDa. In this work, sedimentation velocity analytical ultracentrifugation experiments were performed to obtain *M* for HbGp in oxy and cyano-meta forms. Experiments were performed in Tris-HCl 100 mM containing NaCl 50 mM at pH 7.0, 20°C. HbGp concentration ranged from 50 to 200 μg/mL and speed rotor was 8,000 rpm. The SEDFIT software was used to estimate the $V_{bar}$ =0.755±0.004 mL/mg. The *s** (experimental sedimentation coefficient) value was obtained from SEDFIT program using the value for $V_{bar}$ and, buffer viscosity and density estimated by the Sednterp program. The *s** values were corrected to standard conditions (water and 20 °C) in which, by linear regression extrapolation, supplied that represent the at 0 mg/mL of protein. values for the oxy-HbGp and cyano-meta-HbGp were 58.4±0.3 S and 59.4±0.3 S, respectively. C (M) distribution indicated that M for oxy-HbGp and cyano-meta-HbGp were, 3,400±100 kDa and 3530±50 kDa, respectively. The ratio between *s/D* supplied *M* of 3,560±50 kDa and 3,610±50 kDa, for oxy- and cyano-meta-HbGP, respectively. Moreover, our results suggest that the true value *M* for HbGp is around 3600 kDa, very close to that of homologous proteins. Besides that the cyanomet form seems to be quite stable in solution.

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## 1893-Pos Photothermal Studies of Ligand Photolysis from Two Heme Peroxidases

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**Board B9**

In this report, the results of photoacoustic calorimetry (PAC) studies involving CO photodissociation from Horseradish Peroxidase (HRP) and Soybean Peroxidase (SBP) are discussed. HRP and SBP, contain a heme active site which can oxidize a large diversity of organic and inorganic compounds. The heme group of both HRP and SBP is 5 coordinate, high spin, where His 170 and His 169 are the HRP and SBP proximal ligands, respectively. It also has been demonstrated that HRP has a direct exit channel from the heme active site to the solvent. In addition, SBP can bind a Tris molecule in the distal pocket near the heme group that could potentially regulate ligand binding. Results of PAC indicate a monophasic relaxation for both HRP and SBP subsequent to CO photolysis in phosphate and Tris buffers and with varying concentrations of Tris. The molar volume/enthalpy changes associated with the monophasic decay are similar for both HRP and SBP: ~7mL/15kcal/mol. The results suggest that for both HRP and SBP, the volume change was due to the displacement of CO to the bulk solvent and the enthalpy change was due to the breaking Fe-CO bond. The results also suggest that the binding of a Tris molecule to SBP doesn't affect the energetics of diffusional CO exit from the SBP active site.

## 1894-Pos Circular Dichroism And Oxygen Binding Properties Of Human Adult Hemoglobin With Reversed Heme

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**Board B10**

Human adult hemoglobin (Hb A) reconstituted from apoglobin and hemin consists of the heme orientation isomers, normal and reversed forms which differ by 180° rotation of the heme about the 5, 15-meso axis. The heme rotation causes to interexchange the heme methyl groups at positions 2 and 7 for the vinyl groups at positions 8 and 3, respectively. This will modify the methyl and vinyl peripheral contacts with globin which have been considered to be important for cooperative oxygen binding properties of hemoglobin. We found that recombinant Hb A (rHb A) expressed in *E. coli* showed heterogeneity of components with the intensity of a positive CD band at 260 nm and that it could be resolved into three components (SP-1, SP-2 and SP-3) by SP-Sepharose column chromatography. $^1$H NMR revealed that SP-1 is identical with native Hb A, while SP-2 and SP-3 largely contain the reversed heme isomer in both the α and β subunits, with a content of ~50% and >80% in SP-2 and SP-3, respectively. To examine the effect of the modification of the heme-protein contact on the structure and function of Hb A, we compared

the CD and oxygen binding properties of the three components with those of native Hb A. Native Hb A shows a distinct positive CD band in both the near-UV and Soret regions, but rHb A with reversed heme exhibits a very weak positive CD band at 260 nm and a prominent negative CD band in the Soret region. Cooperativity (Hill's *n*) decreased from 3.18 (SP-1) to 2.94 (SP-2) to 2.64 (SP-3). These results indicate that changes in the heme-globin contact exert significant influences on CD and the cooperative oxygen binding.

## 1895-Pos Two Globins In The *Caenorhabditis Elegans* Genome With Distinct Ligand Binding And Redox Properties

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**Board B11**

Two putative globins from the *Caenorhabditis elegans* genome have been expressed in *E. coli* for study of their electron transfer and ligand binding properties after flash photolysis. One exhibits a high ligand affinity as observed in other common penta-coordinated globin from invertebrate species, while the other exhibits an extremely low affinity for CO and no reversible binding was found with O2. These differences arise from their mechanism of binding since the heme iron of the low ligand affinity globin is strongly hexa-coordinated in the absence of external ligands, probably with the E7 distal histidine; the replacement of this residue is required for binding other ligands. This contrasts with the high ligand affinity globin in which no distal residue is able to compete for heme binding; in this molecule, the Fe-O2 bond is likely stabilized through hydrogen bonding interactions involving the B10 Tyrosine and the E7 glutamine. Interestingly the ferrous bis-histidyl heme of the low ligand affinity globin can transfer one electron to cytochrome c at a rate comparable to that of mitochondrial redox carriers. In the presence of O2 the ferrous heme is oxidized in less than a second, even if the distal histidine is bound by an outer-sphere electron transfer mechanism. These results in the light of the recently discovered hexa-coordinated globins suggest different redox properties and functions between hexa- and penta-coordinated globins.

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## 1896-Pos Changes of Electronic Perturbations of the Heme c Chromophore in Cytochrome c Induced by Thermal Unfolding

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**Board B12**

We measured the temperature dependent visible electronic circular dichroism (ECD) of the ferri- state of horse heart and bovine heart cytochrome c in the B (Soret) band region. To probe concomitant changes of the secondary structure, the respective far UV-ECD spectra were additionally measured with conventional (CCD) and synchrotron radiation circular dichroism (SRCD). The CCD spectra reflect the well known transitions into an intermediate state above 40o C and into the unfolded, disordered state above 70o C. SRCD measurements detail, quantitatively, the secondary structure changes above the unfolded, disordered state temperature. The ECD spectrum of the B-band displays a couplet, which converts to a blueshifted Cotton band at temperatures above 70o C. The disappearance of the couplet at high temperatures is indicative of a reduced band splitting, which results from electronic and vibronic perturbations exerted by the protein environment. The absence of band splitting suggests that non-covalent interactions between heme and protein are substantially reduced in the unfolded state. However, the moderate changes of the B-band and its persisting rotational strength strongly suggest that all cooperative bonds with axial ligands and with Cys 14 and 17 are still intact. This seems to resemble the structural properties of undecapeptide of bovine heart cytochrome c as reported by Blauer et al. Interestingly, the band splitting is not reduced in the intermediate state, which indicates that it does not depend on the distal Ligand. Changes in the rotational strength might reflect increasing distances between the heme and adjacent aromatic residues such as F82. This study shows how the combination of visible ECD and absorption spectra can be used to explore (electrostatic) heme-protein interactions in different states of the protein.

## 1897-Pos Deoxygenation-linked Self-association of Chicken Hb D: Energetics and Modulation by Inositol Hexakisphosphate (IHP)

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**Board B13**

Component D of chicken hemoglobin (HbD) self-associates to form a dimer-of-tetramers (octamer) when deoxygenated. This deoxygenation-linked self-association produces enhanced cooperativity of oxygen binding with a maximal Hill coefficient greater than 4. The self-association constant, $K_a$, for octamer formation is essentially independent of temperature between 5 and 20 °C with an enthalpy of $\Delta H^\circ = 1 \pm 1$ kcal/mol. Thus the self-association is driven by an increase in entropy with $T\Delta S^\circ = 4.4 \pm 1.2$ kcal/mol at 20 °C. Water release accompanying formation of the tetramer-tetramer interface appears to be a likely explanation for this entropy change. The value of $K_a$ is essentially constant between pH 6.5 and 7.4 but decreases ~ 2.5-fold at pH 8.5. This observation precludes involvement at the interface of residues with $pK_a$ values between 6.5 and 7.4. Surprisingly, the allosteric factor, inositol hexakisphosphate (IHP) increases deoxy Hb D self-association ~ 20-fold with a maximal $K_a$ of $2 \times 10^4$ $M^{-1}$ at an IHP to Hb tetramer ratio of ~

20–40. An increase in $K_a$ with IHP implies that IHP binds preferentially to the octamer. A decrease in $K_a$ at higher ratios suggests that a second IHP binding site on the tetramer interferes with octamer formation or alternatively, a high IHP concentration leads to Hb D tetramer-to-dimer dissociation by binding of IHP to the dimer.

*(Supported by NSF grant MCB-0237651)*

## 1898-Pos Human Tryptophan dioxygenase: A Comparison to Indoleamine 2,3 Dioxygenase

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**Board B14**

In contrast to the diverse superfamily of monooxygenases, there are only two classes of hemecontaining dioxygenases in humans. One is tryptophan 2,3 dioxygenase (hTDO) and the other is indoleamine 2,3-dioxygenase (hIDO), both of which catalyze the oxidative degradation of Trp to N-formyl kynurenine. Although hTDO and hIDO catalyze the same reaction, they engage in distinct physiological functions and show distinct biochemical properties. The molecular properties of hTDO, unlike hIDO, have never been explored in the past. Here we report the first structural and functional characterization of hTDO with resonance Raman and optical absorption spectroscopies. We show that the proximal Fe-His stretching frequency ($\varpi$Fe-His) of hTDO is 229 cm-1, 7 cm-1 lower than hIDO, indicating its weaker imidazolate character ascompared to hIDO. In the CO derivative of the L-Trp-bound enzyme, the Fe-CO stretching and C-O stretching frequencies are 488 and 1972 cm-1, respectively, suggesting that L-Trp binds to the distal pocket with its C2-C3 double bond facing the heme-bound ligand, in contrast to hIDO, in which the indole NH group forms an H-bond with the heme-bound ligand. Moreover, the Km values of hTDO for D-Trp and L-Trp are similar, but the *k*cat value of D-Trp is 10-fold lower than that of L-Trp. In contrast, in hIDO, the Km value for D-Trp is 700-fold higher than L-Trp, whereas the *k*cat values are comparable for the two stereoisomers. Taken together the data indicate that the initial deprotonation reaction of the indole NH group in hTDO is carried out by the evolutionarily conserved distal His, whereas that in hIDO is performed by the heme-bound dioxygen; in addition, the stereospecificity of hTDO is determined by the efficiency of the dioxygen chemistry, whereas that in hIDO is controlled by the substrate affinity.

## 1899-Pos Dissolution of Sickle Cell Hemoglobin Fibers and Gels: Mechanisms, Rates and Stochastics

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**Board B15**

Depolymerization rates and mechanisms are important for many linear fibers that involve movement and pathological processes. They are significant for sickle cell hemoglobin (HbS) because passage of polymer that fails to dissolve under the oxygenating conditions in the lungs will facilitate vaso-occlusion in the systemic circulation; and because dissolution rates may be relevant to resolution of vaso-occlusive crises. We showed that of deoxyHbS fiber depolymerization consists of two reactions: monomer loss at fiber ends producing shortening and dissolution times proportional to fiber length (acting alone this is end-depolymerization), and cooperative ligand-nucleated fracture of fibers producing fragments and many ends, resulting in side-end depolymerization that dissolves fibers rapidly in fractions of a second, with vanishing times independent of initial fiber length (Agarwal et al, J. Mol. Biol. *322*:395–412, 2002; Turner et al, Biophys. J. *91*:1008–1013, 2006). We also reported that depolymerization associated with random breakage exhibits stochastic vanishing times as judged by Monte Carlo simulations and an analytically derived expression; and that gels also show a distribution of vanishing times experimentally. In the present work (1) we examine fiber depolymerization progress curves and stochastics experimentally for comparison with analytic and Monte Carlo predictions; (2) consider gel dissolution stochastics, dependent on factors intrinsic to the dissolution process and factors deriving from stochastics of gel formation and structure; (3) examine the effects of gelation rates and hence domain structure and (4) of gel network cross-links on depolymerization rates. If dissolution times *in vivo* vary as a function of stochastics, gel cross-linking and domain structure, and the kinetics of the preceding gelation, the consequences may be significant for sickle cell disease and its potential inhibition. Mechanisms and stochastic properties may also be relevant to the general class of reversible linear biopolymers.

## 1900-Pos CO Photodissociation and Heme Hydration in Sperm Whale Myoglobin as a Function of Amino Acid Substitution at Residues L29 and V68

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**Board B16**

We examine the role water plays in regulating ligand binding in myoglobin, as a water molecule occupying the deoxy distal pocket slows the rate of CO binding by a factor of about ten. We previously showed [R.A. Goldbeck, S. Bhaskaran, C. Ortega, J.L. Mendoza, J. S. Olsen, J. Soman, D.S. Kliger, R.M. Esquerra (2006) Proc. Natl. Acad. Sci. USA *103*, 1254–1259] that we can use the time-resolved changes in the visible absorption bands upon ligand photolysis to monitor heme hydration. We have studied a variety of myoglobin mutants which affect the occupancy of water in the heme pocket of

the protein to better understand the specific mechanism of the effects of water on ligand binding processes in myoglobin. We monitor the kinetics of CO photodissociation and heme hydration in sperm whale myoglobin as a function of amino acid substitution of two highly conserved residues in close proximity to the heme pocket, L29 and V68, to disentangle the direct effects of residue size and hydrophobicity on CO movement into and out of the pocket from their indirect effect on CO rebinding via their influence on heme pocket hydration.

## 1901-Pos Fast Kinetics study of the Reaction of Oxygen with Geobacillus stearothermophilus Nitric Oxide Synthase

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**Board B17**

Nitric Oxide Synthase (NOS) is a heme protein that catalyzes the formation of nitric oxide from L-arginine (L-Arg) and oxygen in a sequential two-step process. In the first step L-Arg and oxygen react to form N-hydroxy-L-Arginine (NOHA), which then reacts with oxygen to form NO and L-Citrulline in the second step. (6R)-5,6,7,8-Tetrahydro-L-biopterin (H4B) is a cofactor of the protein. The reaction mechanism is not well understood because most of the reaction intermediates have very short lifetimes. In this work, stopped-flow experiments were performed to characterize the heme-oxy intermediates of NOS obtained from the thermophilic bacterium Geobacillus stearothermophilus (gsNOS). We found that, depending on the presence of substrates, the cofactor H4B, and the cofactor analog 7,8-Dihydrobiopterin (H2B), the Soret maximum of the O2-bound intermediate varies from 420 to 427 nm, and its decay rate varies from 1s-1 to 15 s-1, at room temperature. We also found that under different cofactor conditions the decay of the oxy intermediate shows some unique thermodynamic properties. Furthermore, in the absence of H4B the oxy-intermediate decays directly to the ferric form; however, in the presence of H4B an additional intermediate is observed.

## 1902-Pos Proximal Ligand Switching Mechanism of iNOS

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**Board B18**

Inducible Nitric Oxide Synthase (iNOS) is one of three isoforms of NOS responsible for the oxidation of L-Arginine to NO and L-citrulline. This isoform is implicated in the pathophysiology of several inflammatory disorders including arthritis, atherosclerosis, and transplant rejection. It is important to know its mechanism of regulation in order to understand the pathophysiology of iNOS. Heme oxygenase-1 (HO-1) which is known to mediate anti-inflammatory effects, is an inducible isoform of the only enzyme in the body known to produce CO. In addition, it has been shown that CO binding to iNOS promotes its gradual conversion to an inactive "p420" form over the course of ~6 hours. On this basis, we hypothesize that the cross-talk between HO-1 and iNOS plays an important role in attenuating the activity of iNOS and modulating inflammatory responses in vivo. Using nanosecond time-resolved Raman scattering, we found that the proximal heme iron ligand of the active enzyme, cysteine, was replaced by a histidine in the p420 form of the enzyme. We show that upon oxidation, the histidine ligand can be replaced with the original cysteine ligand to resume its catalytic activity. The structural properties of the p420 form of the enzyme were further investigated and compared to the active enzyme by steady state resonance Raman spectroscopy. The optical absorbance spectrum of the p420 form of iNOS is similar to that of carboxyhemoglobin in the visible region, which also has a histidine proximal ligand, confirming that histidine is the proximal ligand in the p420 form of iNOS. From these studies, we hypothesize a novel reversible ligand-switching mechanism that may be critical for the in vivo regulation of iNOS activity involving endogenous CO.

## 1903-Pos A Map of the Ligand Diffusion Pathway for Ligand Entry and Exit in Cerebratulus lacteus mini-Hemoglobin

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**Board B19**

Some single domain globins containing TyrB10 are thought to have alternate pathways [1] for ligand entry and exit distinct from the classical, HisE7 gate mechanism exemplified in mammalian myoglobin. Determination of ligand binding and preferential O2 stabilization mechanisms in Cerebratulus lacteus hemoglobin provide insight into the relationship between structure and the evolution of function in animal globins. The hydrogen-bonding network between TyrB10, ThrE11, and GlnE7 in the distal pocket, which destabilizes bound O2, yielding a moderate oxygen affinity (1.3 μM-1), also appears to render the GlnE7 residue immovable [2]. Thus, the E7 amide side chain is inhibited from acting as a gate for ligand diffusion. Instead, an apolar channel appears to act as an alternate pathway for ligand diffusion, allowing for high rates of ligand uptake and release to aid in the rapid delivery of oxygen to neuronal tissue during periods of anoxia [3]. Decreases in association (8-fold) and dissociation (20-fold) rates as well as increases in extent of geminate recombination (16-fold) are observed upon blockade of the channel by mutagenesis of tunnel residues Val7(B6), Ala55 (E18) and Leu86(G12) to Trp. These same kinetic effects of channel blockade by Trp insertions are observed when the distal hydrogen-bond network is abolished by creating a completely apolar distal pocket by TyrB10Phe, GlnE7Leu, and ThrE11Val mutations. Thus,

the interior channel appears to be the primary route of ligand diffusion in Cerebratulus lacteus hemoglobin even when the distal side chains are more apolar and flexible.

## References

[1]. Pesce et al., 2002, Structure *10*, 725
[2]. Pesce et al., 2004, J. Biol. Chem. *279*, 33662
[3]. Vandergon, et al., 1998, J. Biol. Chem. *273*, 16998

# 1904-Pos Nitrite reductase/anhydrase activity of hemoglobin

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**Board B20**

We have shown that hemoglobin acts as an allosterically controlled nitrite reductase, reducing nitrite to nitric oxide (NO) at low oxygen pressures and thereby contributing to hypoxic vasodilation [1–3]. Nitrite reacts with deoxyhemoglobin to form NO and methemoglobin. Maximum NO generation rates occur near the hemoglobin $P_{50}$. Kinetic analysis suggests that NO released from the reaction of nitrite with hemoglobin inside the red cells should not be able to escape heme autocapture. We have discovered that products of the nitrite-hemoglobin reaction generate dinitrogen trioxide ($N_2O_3$) via a novel reaction of NO and nitrite-bound methemoglobin [4]. The O-bound form of nitrite-methemoglobin exhibits ferrous heme nitrogen dioxide ($Fe^{II}$-$NO_2^{\bullet}$) character so it may rapidly react with NO to form $N_2O_3$. $N_2O_3$ partitions in lipid, homolyzes to NO, and readily nitrosates thiols, all of which are common pathways for NO escape from the erythrocyte. These results reveal a fundamental heme-globin and nitrite catalyzed chemical reaction pathway to $N_2O_3$, NO, and S-nitrosothiol, which could form the basis of *in vivo* nitrite-dependent signaling.

## References

[1]. K. Cosby et. al., Nature Medicine, *9*:1498–1505; 2003.
[2]. K. T. Huang et. al., Journal of Biological Chemistry, *280*:31126–31131; 2005.
[3]. Z. Huang et. al., Journal of Clinical Investigation, *115*:2099–2107; 2005.
[4]. S. Basu et. al., Nature Chemical Biology, In Press: 2007.

# 1905-Pos Enzymatic Materials: Kinetic Studies of Microperoxidase-11 Adsorbed onto Zirconium Phosphate Galleires

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**Board B21**

The ability to encapsulate enzymes into a variety of inert materials is important for the development of integrated bio-materials that can serve as highly efficient and selective catalysts. Equally important is understanding the reaction dynamics associated with such hybrid systems. In this report the optical properties as well as reaction kinetics/energetics are probed for a bio-inspired microperoxidase immobilized in αZr phosphate material. Steady state and transient absorption studies demonstrate a high affinity association of MP-11 to Zr-Phosphate galleries resulting in biphasic ligand recombination for CO subsequent to photolysis from the Fe(II) form of the adsorbed MP-11. Photoacoustic calorimetry of the CO release demonstrates significant differences in both molar volume and enthalpies relative to unbound MP-11. These results will be discussed in the context of ligand binding dynamics in immobilized heme protein systems.

# 1906-Pos Nitric Oxide scavenging by plasma hemoglobin

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**Board B22**

The bioavailability of Nitric Oxide (NO), the endothelial derived relaxation factor, can be greatly compromised in vivo by excess amounts of cell-free Hemoglobin (Hb), which are released under pathological conditions such as hemolytic anemias and stroke. The presence of cell-free Hb is controlled by haptoglobin (Hp) which binds cell-free Hb. The complex is then taken up and degraded by macrophages. Previous work has found that Hp type (1-1 or 2-2) affects the outcome of hemorrhagic stroke, perhaps due to differences in NO scavenging. Potentially contributing factors to these differences are uptake rates of cell-free Hb by Hp, inherently different Hb-Hp reaction rates with NO, and rates at which the Hp-Hb complex is taken up by macrophages. We have applied a photolysis technique and a novel competition-sedimentation method to show that there are no significant differences in the NO scavenging rates of Hb bound to Hp1-1 or Hp2-2. We have also performed computational modeling, mimicking conditions found during a stroke, and found that a factor of two difference in Hp-Hb macrophage uptake rates could significantly affect NO bioavailability. Our data thereby support the idea that the rate of Hp-Hb uptake by macrophages (which differs for different haplotypes) contributes to pathology.

## 1907-Pos Heme-Protein Interactions in Horse Heart Ferricytochrome c Induced By Changes of Ionic Strength and Anion Binding to Protein Surface Charges

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**Board B23**

We have measured the absorbance and electronic circular dichroism (ECD) of horse heart ferri-cytochrome c for the Soret and 695 nm charge transfer band as a function of hydrogen phosphate and sodium acetate concentrations. The absorption and ECD profiles of the charge transfer band broaden substantially and the respective integrated intensities increase with rising hydrogen phosphate concentration. Concomitantly, the characteristic couplet displayed by the B-band changes significantly. Increasing the acetate concentration causes a shift and an increase in intensity on the low energy side of the charge transfer absorbance band, whereas the corresponding ECD remains nearly unaffected. In addition, only small changes were obtained for the B-band couplet. Our results indicate that an ionic strength increase (via increasing acetate ion concentration) causes some changes in the Fe-Met80 linkage, probed by the charge transfer band, without a substantial modification of the heme environment. However, the binding of hydrogen phosphate ions causes more significant structural changes, which most likely involve a strengthening of the Fe-Met80 bond, which increases the redox potential. Additional structural variations might also involve Phe82, and the hydrogen bonding network in the heme pocket. In contrast to the current belief of anion binding to the protein's binding domain for cytochrome c oxidase, our results show that the structural variations that occur in the heme pocket are most likely of functional significance.

## 1908-Pos NO Migration and Binding in Myoglobin

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**Board B24**

Fourier transform infrared spectroscopy is a powerful tool for the investigation of protein-ligand interactions in heme proteins. From the variety of ligands that bind to the heme iron, nitric oxide (NO) and carbon monoxide (CO) are particularly attractive, as their bond stretching vibrations give rise to strong mid-infrared absorption bands that can be measured with exquisite sensitivity and precision using photolysis difference spectroscopy at cryogenic temperatures. These stretching bands are fine-tuned by electrostatic interactions with the environment and, therefore, the ligands can be utilized as local probes of structure and dynamics. We have measured infrared spectra of the stretching absorption on NO in the heme-bound and photodissociated states of ferrous and ferric nitrosyl myoglobin (MbNO) and a few site-specific Mb mutants. In the NO-bound state, conformational heterogeneity was inferred from the appearance of multiple bands, arising from different electrostatic interactions with active site residues, most importantly, His64. In ferrous MbNO, a primary photoproduct site was found similar to the B site of MbCO, as indicated by a characteristic NO stretching spectrum. In ferric MbNO, the His64 side chain appears to interfere with trapping of NO in this site; only a very weak photoproduct spectrum was observed in Mb variants in which His64 was present. Upon extended illumination, the photoproduct spectrum changed in a characteristic way, indicating that NO readily migrates to a secondary docking site C, the Xe4 cavity, in which the ligand performs librational motions on the picosecond time scale. This docking site may play a role in the physiological NO scavenging reaction. Surprisingly, NO cannot be trapped at all in secondary docking site D of MbCO, the Xe1 cavity.

**Membrane Proteins - I**

## 1909-Pos Single Cell Studies of Cadherin Adhesion Dynamics

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**Board B25**

The pre-steady state kinetics of cadherin-mediated intercellular adhesion mediated by cadherin was studied by micropipette manipulation measurements. The resulting binding probability versus contact-time profile reveals two binding states. There is a first, fast binding stage that results in a low probability binding state. This is followed by a lag and then slower increase to a high probability binding state. This biphasic profile differs from the simple rise to a limiting plateau predicted for simple binding between proteins via a single binding site. Measurements with the truncated extracellular region of C-cadherin did not change the biphasic kinetic profile. This indicates that the initial binding kinetics is independent of the cytoplasmic domain. Further studies with mutants lacking different domains showed that the third domain (EC3) of the extracellular region is required for both the two-stage kinetic mechanism and the slow forming second, high probability binding state. Mutating the critical Trp2 residue also abolished the two-stage kinetic process. Studies with domain deletion mutants further mapped the fast, first step to the outer two cadherin domains (EC12). This behavior is inconsistent with the assumed binding model for cadherins in which the proteins are postulated to bind via a single binding site in the N-terminal EC1 domain of the extracellular region.

## 1910-Pos Sorting Signal of *Escherichia coli* OmpA is Modified by Oligo (R)-3-Hydroxybutyrate

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