with quantum dots are hydrodynamically stretched and imaged with a TIRF microscope. Activity of the replisome is observed as a change in the DNA length due to the differing force-dependent extension of single- and double-stranded DNA at low pico-Newton forces. We employ a two-color imaging scheme to monitor DNA length in real-time and to stroboscopically image fluorescently labeled single proteins interacting with DNA. Observation of labeled proteins in an ongoing replication reaction allows us to pose structural questions about the stoichiometry and exchange of proteins at the prokaryotic replication fork. We will discuss preliminary results on primer extension by the T7 DNA polymerase and strand-displacement synthesis by the coupled activity of the T7 helicase and polymerase.

2865-Plat

Real-time DNA Synthesis Dynamics Of Single f29 DNA Polymerase Molecules With Base Pair Resolution

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Real-time observation of uninterrupted DNA synthesis by individual f29 DNA polymerase molecules is presented. Four spectrally distinct fluorophores were used to label the four nucleotides via the terminal phosphate moiety, enabling identification of nucleotide incorporation events into the growing DNA strand with base-pair resolution, and correlating these events to the DNA template sequence. Immobilization of polymerase molecules inside zero-mode waveguide nanostructures allowed detection of incorporation events at 100-500 nM nucleotide concentrations, resulting in polymerization rates of ~1-5 bases/s, and lasting for thousands of bases synthesized. Many aspects of the underlying DNA polymerase dynamics are directly observable, such as nucleotide binding, catalysis, pausing, persistence of distinct kinetic states, DNA template sequence context effects, and switching between polymerization and exonuclease activities.

2866-Plat

Anchoring, Sliding, And Rolling: Visualizing The Three-dimensional Nano-motion And Orientation Of A Single Virus As It Diffuses On A Flat Membrane

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The localization of objects within the cell and the accurate measurement of relative positions on the molecular level are essential to an understanding of the function of macromolecular complexes. As a consequence, much effort has been directed towards developing imaging techniques that allow the temporal and spatial resolution of events on the nanoscopic scale. Due to their non-invasive nature, optical techniques are particularly suited for studying live samples and several methods have recently demonstrated subdiffraction resolution. However, all these novel methods are based on detecting fluorescence and thereby face strict limitations in accuracy, time-resolution and dimensionality. Here, we show how the combination of label-free detection of nano-objects and single molecule fluorescence detection allows one to map the center of mass motion and the absolute orientation of a single virus with nanometer resolution in real time. We use interferometric scattering detection to resolve the position of individual virions of Simian Virus 40 (a 45 nm DNA tumor virus) with 2 nm accuracy while bound to its cellular receptor GM1 in supported membrane bilayers. At the same time, we detect the fluorescence of a single fluorescent quantum dot attached to the virus via streptavidin-biotin linkage and determine its position with 4 nm accuracy. By overlapping the fluorescence and scattering trajectories, we can resolve the absolute three-dimensional nano-motion of the virus as it diffuses on a two-dimensional membrane. We find that membrane-bound virions exhibit different modes of motion that are strongly influenced by the concentration of the GM1 receptor in the membrane. Besides Brownian motion in the plane of the membrane, we also observe rolling motion on the sub-20 nm scale and periods of apparent standstill in both two and three dimensions.

Platform AZ: Heme Proteins

2867-Plat

The Single Domain Hemoglobin From Campylobactor Jejuni: The Unique Structural Features Underlying Its NO Dioxygenase Activity

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Recently three groups of hemoglobins (Hbs) have been identified in unicellular organisms: (1) the truncated Hbs (trHb) that display a novel two-over-two alpha-helical structure, (2) the flavohemoglobins (FHb) that comprise a Hb domain with a classical 3-over-3 alpha-helical structure and a covalently attached flavin-containing reductase domain, and (3) the single-domain Hbs (sdHb) that exhibit high sequence homology and structural similarity to the Hb domain of FHb. On the basis of phylogenetic analysis, the trHbs can be further divided into three subgroups: TrHb-I, TrHb-II, and TrHb-III. C. jejuni contains two globins, a single domain hemoglobin, Cgb, and a truncated hemoglobin, Ctb. Neither Cgb nor Ctb are required for the survival of the bacterim in air. Cgb knock-out mutant cells are hypersensitive to reactive nitrogen species, while Ctb knock-out cells do not display any sensitivity to nitrosative stress. As the expression of Cgb is induced by nitrosative stress, it is believed to function as a NO dioxygenase to protect C. Jejuni against the toxic effects of NO. In contrast, Ctb is thought to be involved in regulating O2 flux into and within the cell. To study the structural and functional properties of Cgb, we have purified the recombinant Cgb protein expressed in E. coli. Due to its high affinity towards cyanide, Cgb is isolated in the cyande-bound ferric state. By using resonance Raman scattering and fast kinetic techniques, we have studied the structural, functional and ligand binding properties of Cgb, with respect to Ctb as well as other globins. The implications of these data will be discussed in the context of the NO dioxygenase chemistry carried out by this fascinating globin.

2868-Plat

Two distinct functional globin classes in *Caenorhabditis elegans*

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The nematode *Caenorhabditis elegans* expresses 33 different globin genes. We studied the expression of two of them, *glb-1* and *glb-26* in more detail using green fluorescence protein technology and found that both are expressed in distinct subsets of cells. GLB-1::GFP is mainly observed in head and tail muscular or hypodermal tissue and in a subset of nerve cells. GLB-26::GFP is seen in the head mesodermal cell and in stomato-intestinal muscle. Hypoxia causes upregulation of the expression of *glb-1,* but not *glb-26.* After expression in an *E. coli* system purified GLB-1 and GLB-26 were spectroscopically (UV/VIS, resonance Raman, electron paramagnetic resonance) and kinetically characterized. The 3D structure of GLB-1 was determined.

GLB-1 is pentacoordinated and exhibits high affinities for O_2 and CO. The bound O_2 is stabilized through hydrogen bonding interactions involving a B10 tyrosine and an E7 glutamine. In contrast, in absence of ligands, GLB-26 is strongly hexacoordinated with E7 distal histidine as sixth ligand. In the presence of O_2, this globin is instantly oxidized to the ferric form and is therefore incapable of reversible oxygen binding. Most likely this molecule will function as an electron transfer protein.

Taken together these data strongly suggest that GLB-1 and GLB-26 belong to two globin classes with totally different functions. Potential functions will be discussed.

2869-Plat

Peroxidase Activity Of Respiratory Proteins. The Role Of Protein Bound Free Radicals

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Peroxidase activity of respiratory haem proteins is associated with formation of two active species on the protein when it reacts with peroxides: the oxoferryl state of the haem and a protein bound free radical. Both the free radical and the oxoferryl haem are capable of oxidising a range of substrates. General principles of formation of these two species and of their further reactions will be considered. Experimental confirmation of the proposed view involves a complex analysis of diverse pathways by which electrons can be passed between different parts of the protein. Associated with this electron transfer, the process of free radical character transfer around the protein as well as between different protein molecules can be monitored by the EPR spectroscopy. If a protein contains tyrosine residues (which is often but not always the case), the chance of observing a tyrosyl radical is high. EPR data will be presented on formation, transformation and transfer of protein bound radicals in different respiratory proteins. A method will be described that allows very accurate determination of the tyrosyl radical parameters. By using the method, it is possible to determine the three principal g-values of the radical solely from an X-band EPR spectrum. (And if a high field EPR spectrum of the radical is available, and the g-values are measured directly, the other radical parameters, e.g. the hyperfine interaction constants, can be determined much more accurately.) The radicals parameters extracted from experimental EPR spectra are then compared with either some

structural features of the tyrosines (phenoxyl ring rotation angle) or with the values of the parameters obtained from the DFT calculation. This allows identification of the tyrosine in the protein responsible for the observed radical.

2870-Plat

Archaeal Protoglobin Structures: Novel Ligand Diffusion Paths And Heme Reactivity Modulation

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Despite its strict anaerobic nature, M. acetivorans genome hosts genes related to O2 metabolism; among these, an open reading frame encodes for a ''protoglobin'' (NP_617780; Pgb). Pgbs are single domain heme proteins of ~195 amino acids, related to the N-terminal domain of archaeal and bacterial globin coupled sensor proteins (GCS; Freitas et al. (2004) Proc. Natl. Acad. Sci. USA 101, 6675-6680). Sequence comparisons indicate that Pgbs, despite their 30-35% larger size, are structurally related to single chain hemoglobins (about 150 amino acids, 3-on-3 α-helical sandwich,12-16% residue identity to Pgbs), and to the heme-based aerotaxis transducer sensor domain of Bacillus subtilis GCS. Pgbs bind O2, CO, and NO reversibly in vitro. Functional and evolutionary issues are openly debated: Pgb may facilitate O2 detoxification in vivo promoting electron transfer to O2, or may act as CO sensor/supplier in methanogenesis.
Our previous studies unravelled the 3D structure of M. acetivorans Pgb, and of its ligand binding properties (Nardini et al. (2008) EMBO Reports 9, 157-163). We showed that Ma-Pgb heme- domain is strongly related in tertiary and quaternary structure to the N-terminal domain of archaeal and bacterial GCSs. Contrary to known globins, however, Pgb-specific loops and a N-terminal extension completely bury the heme within the protein matrix. A new access routes to the heme, built by two Pgb-specific apolar tunnels reaching the heme distal site from locations at the B/G and B/E helix interfaces was highlighted. We present here structural and ligand binding properties of four Ma-Pgb mutants (at sites B10, B12, G8, G11) that were designed in order to probe the role of the heme access tunnels previously described. The atomic resolution structures will be discussed at the light of the kinetic parameters measured for the mutant Ma-Pgb.

2871-Plat

Structural Probes Of Reactive Intermediates Of Dehaloperoxidase From Amphitrite ornata

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The enzyme dehaloperoxidase (DHP) from the marine worm Amphitrite ornata is a unique hemoglobin that functions as a peroxidase, capable of converting 2,4,6-trihalo- phenols (TBP, TCP, and TFP) into the corresponding 2,6-dihalogenated quinones as well as other products. In this overview talk we discuss the structure and function of DHP using X-ray crystallography and nuclear magnetic resonance (NMR) to discuss the large differences between DHP function and hemoglobin function despite the strong structural similarities. The position of halogenated phenols inside the distal pocket is one anomalous feature of DHP that is not observed any other hemoglobin or myoglobin. The X-ray crystal structure of DHP reveals that the distal histidine is flexible and has two major conformations. The closed conformation (named by analogy with Sperm Whale myoglobin) is enforced by the binding of a sixth ligand to the heme iron. In the open conformation, observed in the deoxy DHP X-ray crystal structure, the distal histidine is in a solvent exposed conformation. The role of the histidine in coupling the binding of substrate and inhibitors will be discussed in the context of the mechanism for formation of compound I, compound II and a novel intermediate called compound RH that appears to be crucial to the cycling of DHP between hemoglobin and peroxidase function. The NMR data reveal that there are both interior and exterior binding sites for the substrate. This aspect will be discussed along with evidence from optical and EPR spectroscopy to understand the electron transfer kinetics of DHP.

2872-Plat

Structural Analysis of Hemoglobins and Myoglobins Using MD Simulations

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In the family of respiratory proteins, hemoglobins and myoglobins have been the first to be crystallized in '50. Despite their precise 3D structures are available at high resolution, some questions regarding the microscopic functioning remain yet open. The R to T switching mechanism in hemoglobins and the ligand escape process in myoglobins remain still under debate.
Thanks to the small size, myoglobin is the preferred candidate also for the more general structure-funcion paradigm. In the interior of myoglobin five main docking sites have been identified, especially with Xe NMR, and for long time these Xenon cavities have been classified as packing defects. Recently, it was shown that they might be involved in ligands migration path, even if mechanisms used by myoglobin to connect these cavities is still unknown as well as processes regulating its biologic functions. In this work we made use of standard MD simulations of solvated myoglobin to characterize internal cavities. Our principal results is that we have found several secondary cavities showing volume and occurrence at least comparable to that of Xenon cavities. In order to rationalize and in-depth analyze such a huge amount of data (ca. 30000 cavities/10 ns MD), special cluster-analysis was applied: we classified all cavities with respect to the position, size and occurrence as function of simulation time ascribing them to different clusters. This analysis implicitly highlights possible ligand migration paths for small ligands within the protein matrix allowing to quantitatively compare dynamical behaviour of different myoglobins towards different ligands. Our suggestion that the secondary cavities constitute the preferred path for ligand escape is also supported by explicit metadynamics simulations of ligand escape.

2873-Plat

Auto-Oxidation of Human Hemoglobin and the Roles of Distal Heme Pocket Substitutions

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This study investigates the auto-oxidation reaction of human normal adult hemoglobin (Hb A) and the effects of distal heme pocket substitutions, P_{50}, and tetramer-dimer dissociation on the rate and mechanism of auto-oxidation. Recombinant hemoglobins (rHbs) with single amino acid substitutions at helical positions E11 and B10 have been expressed in *Escherichia coli* and purified, as well as di-α linked and octameric rHbs. These rHbs include: rHb (αV62L), rHb (αV62I), rHb (βV67L), rHb (βV67I), rHb(αL29W), rHb(αL29F), rHb(α-Gly-α/β gene di-α/β), rHb(diαL29F), rHb(diαL29W), and rHb(βG83C). Auto-oxidation measurements were conducted with 32μM heme in MES buffer (pH 6.5) for 60 hours at 35 °C. A monophasic nature of auto-oxidation has been observed for Hb A and a biphasic nature for all other rHbs. In comparison to the other mutants, including di-α linked and octameric rHbs, rHb (αL29F) is most resistant to oxidation and rHb (αL29W) is the least resistant to oxidation. Characterization of three novel rHbs; (βL28F, βV67I), (αL29F, βV67I), (αL29F,αV62I) will test whether the fast and slow phases of the observed biphasic nature of auto-oxidation can be attributed to the mutated and wild-type subunits, respectively. Our studies will also provide new insights into the roles of amino acid residues in the distal heme pockets on the structure-function relationship in hemoglobin(Supported by NIH grants HL-024525 and GM-084614, HHMI, and The Arnold and Mabelle Beckman Undergraduate Research Scholars Program).

2874-Plat

The Use Of Glassy Matrices To Identify Intermediates In The Nitric Oxide Dioxygenase Reaction Of Hemoglobins And Myoglobins

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The nitric oxide dioxygenase (NOD) reaction of nitric oxide with ferrous oxy derivatives of Hb and Mbs is important from many physiological standpoints. Several mechanisms have been proposed; however, the experimental difficulties in probing this reaction in solution at physiological pH and ambient temperatures have precluded an unambiguous determination of the sequence and nature of intermediates. We have developed a method of following the progression of this reaction in glassy matrices that allows for the trapping and probing of key intermediates. The technique is based on incorporation of O_2 derivatives of Hb and Mb in a thin glassy matrix (derived from trehalose) that lines the inner wall of an optical quality tube. After purging the sample with dry nitrogen to remove the unbound excess oxygen, the tube is filled with NO. Absorption spectroscopy is used to follow the spectral progression initiated as the NO slowly accesses the heme bound oxygen. The spectra reveal an intermediate that resembles the spectrum attributed to the bound peroxynitrite intermediate. The final product under these condition is a species with a spectrum that is identical to that which is generated when met Hb(Mb) is incorporated into a glass in the presence of an excess of nitrate. The spectrum attributed to the nitrate