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

product only appears when the glass is dry. The results are consistent with water being an effective competitor for the ferric heme site in the presence of nitrate.

Platform BA: Calcium Signaling Pathways

2875-Plat

Isoform-specific Regulation Of The Ca-sensitive Transcription Factor NFAT In The Cardiovascular System

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NFAT transcription factors (Nuclear Factor of Activated T-cells) mediate Ca-sensitive gene transcription and are involved in cardiovascular remodelling. Nuclear localization of NFAT is dynamically regulated by intracellular Ca signals yielding to dephosphorylation and nuclear translocation of NFAT, and activity of intracellular kinases that induce nuclear export. The aim of this study was to analyze the regulation of NFAT in vascular endothelial cells and adult cardiomyocytes. Subcellular distribution of NFAT-GFP fusion proteins (isoforms NFATc1 and NFATc3) was analyzed with confocal microscopy and intracellular Ca ($[Ca]_i$) was measured simultaneously using rhod-2. In calf pulmonary arterial endothelial (CPAE) cells, application of ATP (5 μM) induced nuclear localization of both isoforms (quantified as an increase in $NFAT_{NUC}/NFAT_{CYT}$ ratio). Subsequent attenuation of $[Ca]_i$ to facilitate nuclear export resulted in substantial export of NFATc3 to the cytoplasm, which was sensitive to Leptomycin B (40 nM). Previously translocated NFATc1 was only partially affected by nuclear export, indicating isoform-specific regulation of NFAT in endothelial cells.

In cardiac myocytes regulation of NFAT was isoform-, and tissue-specific: NFATc1 displayed nuclear localization in quiescent myocytes, which was dependent on $[Ca]_i$ and further enhanced by blocking nuclear export (Leptomycin B) or by inhibition of intracellular kinases (20 mM LiCl, 1 μM alsterpaullone or 1 μM SP600125). In contrast, NFATc3 was distributed in the cytoplasm of quiescent cells. Incubation with Leptomycin B, but not inhibition of nuclear kinases induced nuclear localization of NFATc3 in ventricular cells. Incubation with the G_q protein-coupled receptor agonists endothelin-1 (100 nM) and Ang II (2 μM) induced nuclear localization of NFATc3 only in atrial, but not ventricular cells. We conclude that (i) regulation of nuclear NFAT in the cardiovascular system is isoform- and tissue specific and (ii) dynamically regulated by activity of nuclear export pathways.

2876-Plat

Alterations In Binding Properties Of Myocardial Nuclear Membrane Receptors Induce Nuclear Calcium Overload In Rat Ischemia-reperfusion

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Cell nuclei possess an independent calcium regulatory system consisting of nuclear Ca^{2+}-ATPases (NCA), IP_3 receptors (IP_3R), IP_4 receptors (IP_4R), ryanodine receptors(RyR), and nuclear pore complexes(NPC). We studied the changes in $Ca^{2+}{}_n$ and its regulatory system in rat model of myocardial ischemia-reperfusion injury(IRI) induced by 30 min coronary occlusion followed by 180 min reperfusion. The $Ca^{2+}{}_n$ content in isolated nuclei was measured with atomic absorption spectrophotometer. NPC permeability was assessed through the amount of calmodulin conjugated Alexa FluoTM 488 as fluorescent probes. NCA activity was evaluated by phosphate group released from ATP in enzymatic reaction. The maximum binding capacity(B_{max}) and dissociation constant(K_d) of IP_3R, IP_4R and RyR were determined by radioligand binding of [^{3}H]IP_4, [^{3}H]IP_4 and [^{3}H]-ryanodine to isolated cardiomyocytic nuclei. All results are shown in the table. Our findings suggest that in vivo rat myocardial IRI is characterized by $Ca^{2+}{}_n$ overload, upregulations of nuclear IP_3R and IP_4R, downregulations of NCA activity and RyR, and an increase in permeability of NPC. The upregulation of nuclear IP_3R and IP_4R may be responsible for the nuclear calcium overload.

2877-Plat

ATP-evoked Ca2 ± waves Stimulate Gene Expression In Human Airway Fibroblasts

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We sought to investigate the effects of a variety of autacoids on Ca^{2+}-handling in human airway fibroblasts. Primary cultured fibroblasts were loaded with the Ca^{2+}-indicator dye fluo-4 and studied using confocal fluorimetric microscopy. ATP (10^{-5} M) evoked recurring Ca^{2+}-waves . This fluorimetric change was greater and longer lasting within the nucleus of the cell than in the non-nuclear portion of the cytosol, and was only sometimes accompanied by a contraction. These responses were completely occluded by cyclopiazonic acid (10^{-5} M; depletes the internal Ca^{2+}-store) or the phospholipase C inhibitor U73122 (10^{-6} M). Pretreatment of the cells with ryanodine (10^{-5} M), on the other hand, had no effect on the ATP-evoked responses. With respect to the receptor through which this response was exerted, we found it to be mimicked by UTP or ADP but not by adenosine or α,β-methylene-ATP, and to be blocked by the purinergic receptor blocker PPADS; interestingly, PPADS itself appeared to sometimes evoke a rise in $[Ca^{2+}]_i$ on its own. ATP also evoked a membrane conductance change with characteristics of a non-selective cation current, markedly enhanced synthesis of the cytokine TGFβ and the matrix proteins fibronectin and collagen I; these changes in protein synthesis were blocked by PPADs and were partially reduced by ryanodine. We conclude that, in human pulmonary fibroblasts, ATP acts upon P2Y receptors to liberate internal Ca^{2+} through ryanodine-insensitive channels, leading to a Ca^{2+}-wave which courses throughout the cell and triggers protein synthesis.

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2878-Plat

Store-operated Ca^{2+} Entry Is Suppressed During Mitosis Due To Phosphorylation Of The Endoplasmic Reticulum Ca^{2+} Sensor Stim1

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When ER Ca^{2+} stores are depleted due to physiological Ca^{2+} release or pharmacological perturbation, Ca^{2+} influx via plasma membrane Ca^{2+} channels is activated by a process known as store-operated Ca^{2+} entry (SOCE). The current associated with SOCE is Ca^{2+} release-activated Ca^{2+} current (I_{crac}). SOCE involves Orai Ca^{2+} influx channels and STIM ER Ca^{2+} sensors. When ER Ca^{2+} stores are full, STIM1 is localized throughout the ER membrane; however, ER Ca^{2+} store depletion induces rearrangement of STIM1 into punctate structures near the plasma membrane where it activates Orai channels. Interestingly, mitosis is the only known physiological situation in which Ca^{2+} store depletion is dissociated from SOCE or I_{crac} activation. Identification of the molecular components of the SOCE signaling pathway has facilitated analysis of the mechanism underlying mitotic SOCE suppression. We found that in mitotic HeLa cells, an enhanced yellow fluorescent protein-tagged STIM1 (eYFP-STIM1) did not rearrange into puncta in response to Ca^{2+} store depletion and accordingly, SOCE was not activated. We hypothesized that mitosis-specific phosphorylation of STIM1 may underlie the block of STIM1 rearrangement and SOCE suppression. To this end, the phospho-specific MPM-2 antibody recognized eYFP-STIM1 immunoprecipitated from mitotic but not interphase cells. MPM-2 recognizes phosphorylated serine or threonine followed by proline, and human STIM1 contains 10 instances of S/T-P, all located in the cytoplasmic, C-terminus. STIM1 truncation mutants indicate that at least 2 sites within the C-terminus account for the mitosis-specific phosphorylation. Individual phosphorylation site mutants are being created to identify specific phosphorylated residues and to determine the functional consequences of phosphorylation during mitosis. Suppression of SOCE during mitosis may be an important signaling event, because mitotic processes such as chromosome separation and cytokinesis are exquisitely sensitive to small changes in cytoplasmic Ca^{2+}.

2879-Plat

Heteromeric channel assembly of Orai1 and Orai3 exhibits altered Ca2+ selectivity

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Coexpression of STIM1, targeted to the endoplasmic reticulum and each of the three Orai (also termed CRACM) channels located in the plasma-membrane leads to store-operated, highly Ca2+ selective currents. While Orai1 has been reported to form the native Ca2+ release activated Ca2+ (CRAC) channels in human T-cells, the molecular architecture of less Ca2+ selective store-operated currents remains unknown. Here we show employing confocal fluorescence resonance energy transfer (FRET) that all three Orai proteins are able to form homo- and hetero oligomers. Overexpressed homomeric Orai1 or Orai3 together with STIM1, resulted in store-operated inward rectifying, highly Ca2+ selective currents, as resolved by whole-cell patch-clamp recordings. Coexpression of Orai1 together with Orai3 and STIM1 yielded similar store-depletion activated Ca2+ currents, yet with a leftward shifted reversal potential, pointing to less selective currents. In line, a tandem construct where Orai1was linked to Orai3 exhibited a similarly reduced Ca2+ selectivity that allowed for robust Cs+ permeation. Moreover, Orai3 pore mutants coexpressed with wild-type Orai3 affected Ca2+ and Cs+ selectivity/permeability. These