detected, in contrast to the native enzyme whose intrinsic fluorescence markedly increased. This indicates a relatively high rigidity of the substrate-induced enzyme compared with the native protease. It also indicates that the "induced fit" theory is not an adequate explanation of the mechanisms involved in such reactions. Utilising a "lock and key" mechanism for such secondary reactions may have adaptive value in that it facilitates high efficiency in enzymatic reactions.

2332-Pos

Peculiar Regulatory Role of Magnesium in Nucleotide Hydrolysis of dUTPases

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The dUTPase enzymatic activity is indispensable to efficiently reduce cellular dUTP/dTTP levels. Lack of the enzyme leads to erroneous uracil incorporation into DNA resulting in chromosome fragmentation and cell death. dUTPase is therefore reported to be a preventive DNA repair factor and a high-potential drug target in cancer. Although divalent metal ions are indispensable to the catalytic activity of numerous nucleotide hydrolyses, the increase in dUTPase steady-state activity is only twofold in the presence of magnesium. We had specific interest in investigating the influence of magnesium on the catalytic mechanism and the structure of human dUTPase, which is a completion of our previous study revealing the fundamental steps of the enzymatic cycle and providing a quantitative model for the mechanism. To address the above issue, a broad array of techniques were employed, such as transient kinetics, crystallographic and spectroscopic methods. We revealed that the homotrimeric human dUTPase has two structural metal-binding sites within the central chanel of the enzyme with different binding affinities toward the magnesium ions. At the active sites, magnesium facilitates the formation of the catalitically competent gauche conformation of the alpha-phosphate group allowing the nucleophilic attack of catalytic water on the alpha-phosphorus atom. According to our current observations, the steady-state activity monitored in the absence of magnesium is a result of at least two parallel reaction series. One reaction pathway is consistent with our previous model of dUTPase catalysis and occurs very slowly without magnesium. The other possible pathway potentially involves hydrolysis initiated by nucleophilic attack on the beta-phosphorus atom.

2333-Pos

Conservation of Active Site Geometry in Evolution of Iron Lipoxygenases: EPR Studies

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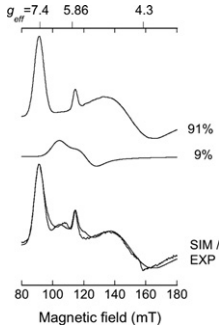
Lipoxygenases employ a redox-active metal center (Fe or Mn) in electron-proton coupled reaction to initiate oxidation of unsaturated fatty acids. In this study, the iron center geometry is examined by EPR for two newly characterized bacterial lipoxygenases, and the data are compared with similar studies of the eukaryotic lipoxygenase-1 from soybean (Glycine max). Although the protein sequences of bacterial lipoxygenases (from Pseudomonas aeruginosa and Shewanella woodyi) are only 27 and 20% identical, respectively, to the soybean sequence, the sequences are highly similar in regions known to contribute side chains to active site cavities and to metal binding in the soybean protein. Remarkably, all three lipoxygenases reveal an identical set of iron EPR sub-spectra, but rates of inter-conversion of the sub-spectra differ. Multiple sub-spectra are also seen in ferrous-NO enzyme forms. Immediately after lipoxygenase iron is activated from ferrous to ferric, predominantly one of the EPR sub-spectra is observed, and this intermediate converts to multiple sub-spectra with time (minutes for soybean, longer for bacterial forms). The rate of formation of the first activated ferric state, the enzyme kinetics lag, and products of single substrate turnovers are compared.



2334-Pos

The N-Terminal Ig Domain of Endoglucanase Cel9A from the Thermoacidophilic Alicyclobacillus Acidocaldarius Enhances Protein Stability

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As part of our ongoing studies of thermophilic cellulases, we are examining the subfamily E1 of family 9 of glycoside hydrolases, members of which have an N-terminal immunoglobulin (Ig)-like domain followed by the catalytic domain (CD). While the function of the Ig-like module has not been determined, deletion of the Ig-domain results in complete loss of enzymatic activity in the cellobiohydrolase, CbhA from *Clostridium thermocellum*. In this work we used simulation approaches to investigate the role of the (Ig)-like domain in the non-processive endonuclease Cel9A from the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* (*Aa*_Cel9A) for which the crystal structure has only recently been resolved. We are using molecular dynamics (MD) simulations to provide a dynamic view of *Aa*_Cel9A. Our goal is to try to piece together the available activity, kinetic, biophysical and structural information and to offer insights into domain interactions and domain motions that may be associated with *Aa*_Cel9A activity and stability. To examine the role of the Ig-domain, MD simulations combined with a simplified force field model were performed on the structure of *Aa*_Cel9A both with and without the Ig-like domain. Umbrella Sampling and free energy perturbation (UM/FEP) were also performed to obtain unfolding free energy landscapes for both cases. Both methods show that the Ig-like domain stabilizes the structure of the catalytic domain; thus, a major function of N-terminal Ig-like domain appears to be to confer thermostabilty. We also used the results of our MD simulations to study correlated motions among atoms in the Ig-like domain and atoms in the CD. Our preliminary results show that Ig-like domain motions are correlated with active site molecular motions, suggesting that the Ig-domain may be required for proper control and orientation of active site residues.

2335-Pos

First-Principles Study of Non-Heme Fe(II) Halogenase SyrB2 Reactivity

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We present here a computational study of reactions at a model complex of the SyrB2 enzyme active site. SyrB2, which chlorinates L-threonine in the syringomycin biosynthetic pathway, belongs to a recently discovered class of α-ketoglutarate, non-heme Fe(II)-dependent halogenases that shares structural and chemical similarities with hydroxylases. Halogenases and hydroxylases alike decarboxylate the αKG co-substrate, facilitating formation of a high-energy ferryl-oxo intermediate that abstracts a hydrogen from the reactant complex. The reaction mechanisms differ at this point, and mutation of active site residues fails to reproduce hydroxylating activity in SyrB2 or halogenating activity in similar hydroxylases. Using a density functional theory (DFT) approach with a recently implemented Hubbard U correction for accurate treatment of transition-metal chemistry, we explore probable reaction pathways and mechanisms via a model complex consisting only of the iron center and its direct ligands. We show that the first step, αKG decarboxylation, is barrierless and exothermic, while the subsequent hydrogen abstraction step has an energetic barrier consistent with that accessible under biological conditions. In the model complex we use, radical chlorination is barrierless and exothermic, while the analogous hydroxylation is found to have a small energetic barrier. The hydrogen abstraction and radical chlorination steps are strongly coupled: the barrier for the hydrogen abstraction step is reduced when carried out concomitantly with the exothermic chlorination step. Our work suggests that the lack of chlorination in mutant hydroxylases is most likely due to poor binding of chlorine in the active site, while mutant halogenases do not hydroxylate for energetic reasons. While secondary shell residues undoubtedly modulate the overall reactivity and binding of relevant substrates, we show that a small model compound consisting exclusively of the direct ligands to the metal can help explain reactivity heretofore not yet understood in the halogenase SyrB2.

2336-Pos

Pre-Steady-State Kinetic Analysis of the Elongation Mode of Dengue Virus RNA Polymerase Domain

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Dengue viral RNA polymerase replicates its positive single-stranded RNA genome in a primer-independent manner. The slow and inefficient initiation during replication masks the elongation mode. The aim of this work was to further characterize the mechanism of elongation towards an increased understanding of how the enzyme selectively recognizes different nucleotides. Transient kinetic methods were used to measure the microscopic rates of the reaction pathway comprised of enzyme and RNA binding followed by nucleotide binding and incorporation. After extended pre-incubation of the enzyme with double stranded RNA (12-mer primer with a 26-mer template), addition of a correct nucleotide resulted in a burst of single nucleotide incorporation,

and the amplitude of the reaction was used to monitor the time course of RNA binding. The kinetics of enzyme and RNA binding followed a two-step mechanism: an initial binding was followed by a conformational change. The enzyme and RNA binding proceeded to equilibrium about six times faster at 37 oC than at 30 oC, suggesting the conformational change involved in RNA binding is temperature sensitive. Following incubation of RNA and enzyme to form an active complex, we used chemical quench-flow methods to measure the ground state binding K_d and the incorporation rate of a correct nucleotide. The fidelity of the polymerase was determined by measurement of the incorporation of incorrect nucleotides. Using these methods, we characterized the Dengue polymerase in its elongation mode by measuring the kinetics of RNA and polymerase binding and the kinetics for nucleotide binding and incorporation. Based on these data, we built a working model for studying the selectivity of Dengue polymerase.

2337-Pos

Structure-Guided Design of Novel Inhibitors of Human Uridine Phosphorylase 1

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Uridine phosphorylase (UPP) is a key enzyme of pyrimidine salvage pathways, catalyzing the reversible phosphorolysis of ribosides of uracil to nucleobases and ribose 1-phosphate. It also plays a role in the activation of pyrimidine-based chemotherapeutic compounds such a 5-fluorouracil (5-FU) and its prodrug capecitabine. In some cases, an elevated level of this enzyme in solid tumours is thought to contribute to the selective action of these drugs. Nevertheless, the therapeutic value of these fluoropyrimidine antimetabolites is often limited by their toxicity to normal tissue. To address this shortcoming, specific inhibitors of UPP, such as 5-benzylacyclouridine (BAU), have been clinically studied for their ability to moderate the cytotoxic side effects of 5-FU and its derivatives, so as to improve the therapeutic index of these agents. We have determined the high resolution structures of human uridine phosphorylase 1 (hUPP1) in complex with natural ligands and known inhibitors. The structures reveal important details underlying the architecture of hUPP1's active site and the proximate surfaces that influence binding of BAU and analogous acyclouridine compounds. This data provides opportunities for designing more potent inhibitors of this enzyme. For instance, the back wall of the substrate binding pocket is conformationally unique relative to earlier elucidated structures of microbial homologues of UPP. These features can be exploited to develop novel inhibitory compounds with improved efficacy against the human enzyme as a step toward the development of better chemotherapeutic regimens that protect normal tissues with relatively lower UPP activity.

Protein Structure II

2338-Pos

Factor Xa Dimerization and Prothrombinase Complex Formation are Competitive Process on a Membrane Surface

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Exposure of phosphatidylserine (PS) molecules on activated platelet membranes is a crucial event in blood coagulation. Binding of PS to a specific site on factor Xa (fXa) and factorVa (fVa) promotes their assembly into a complex that dramatically enhances proteolytic activity of fXa. Recent studies demonstrate that by both soluble PS and PS-containing membranespromote formation of inactive fXa dimer at 5mM Ca^{+2}. The fluorescence anisotropy of active site labeled fXa, FEGR (Fluorescein-GLU-GLY-ARG-chloromethylketone)-Xa, is decreased in the presence of PS membrane on which it forms dimer. We report now the addition of fVa to membrane-bound FEGR-Xa produced fVa- FEGR-Xa complex formation with a $K_{d,\ surface}$ approximately 60-fold lower than that characterizing FEGR-Xa surface dimerization, clearly indicating fVa strongly competed with fXa dimer formation in order to form active Xa-Va complex on the membrane surface. Analysis of FEGR-Xa fluorescence anisotropy yielded roughly constant K_d's for Xa-Va interaction with increasing Ca^{2+} concentration from 2 to 5 mM Ca^{2+} despite the fact that fXa dimer formation varied dramatically over this Ca^{2+} range. Experiments performed at varying membrane and fVa concentrations for both 23 nm and 120 nm confirmed that protein distribution between vesicles was sufficiently rapid as to overcome any possible effects of membrane discreteness. We conclude that PS-induced fXa dimerization on membrane strongly competes with fXa-fVa complex formation at high Ca^{2+} concentrations. Supported by USPHS grant HL072827 to BRL.

2339-Pos

Sans and Osmotic Stress Approach to Study Protein Preferential Hydration and Association

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The function of biological macromolecules necessarily depends on their hydration and interactions. However, it can be challenging to appropriately and directly measure these forces. We are using a combined small-angle neutron scattering (SANS) and osmotic stress approach to directly correlate protein structure and structural transitions with the associated hydration and energetics. We performed SANS experiments on hexokinase (HK) to investigate protein preferential hydration by solute molecules, called osmolytes, and the interactions responsible for HK dimer formation. The HK monomer-dimer equilibrium plays a regulatory role but its importance to function is not entirely clear. With SANS, three regions of scattering contrast are created upon osmolyte addition: protein, protein-associated water, and bulk water/osmolyte solution. Changes in the zero-angle scattering intensity, $I(0)$, and the apparent radius of gyration, R_g, with increasing osmolyte concentration are used to quantify the number of osmolyte-excluding water molecules associated with protein. We observe the preferential hydration of HK monomer and dimer states to depend on the osmolyte chemistry and size but find the calculated hydration change accompanying the monomer-dimer transition to be independent of the osmolyte used. By experimentally exploring the forces that are important for guiding protein association, we hope to address critical questions concerning protein structure, hydration, and interactions.

2340-Pos

Understanding the Effects of Molecular Crowding on the Structure and Stability of Proteins Using NMR Spectroscopy

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Much of the research on biological proteins is performed *in vitro* (under artificial conditions) involving the isolation of the protein from the cell. The living cell, however, constitutes a very complex system, and a protein's structure and stability may be influenced by its native environment. An understanding of the effects of molecular crowding will provide important information regarding a protein's structure, dynamics, and stability *in vivo*.

This study involves the titration of ^{15}N FGF-1 with various intracellular components (to simulate an in-cell environment) followed by NMR spectroscopy to determine any chemical shift perturbation corresponding to shifting amino acid residues. Fibroblast Growth Factor 1 (FGF-1) is a protein involved in cellular proliferation, wound healing, and cancer development and metastasis. Little information is known regarding FGF-1's interactions inside the cell, as it follows a non-classical secretion pathway. To better understand the role of intracellular proteins on the structure of FGF-1, several experiments were carried out using multi-dimensional NMR spectroscopy; to the FGF-1 sample were added (1) intracellular proteins (from the purification of unlabeled FGF), (2) intracellular proteins and lysozyme, and (3) lysozyme alone (added as a control). HSQC data was obtained at regular intervals and processed using XWIN-NMR and Sparky software. A chemical shift perturbation plot was constructed from the data to show the (individual and combined) effects of the addition of the intracellular proteins and/or lysozyme on FGF-1. The preliminary results of this study indicate that a moderate number of amino acid residues were perturbed with the addition of intracellular proteins. This implies that molecular crowding plays a role in the structural conformation of FGF-1 and possibly other proteins *in vivo*.

2341-Pos

Anthrax Protective Antigen Oligomerization Regulates Toxin Activity

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Anthrax toxin (Atx) is a key virulence factor secreted by Bacillus anthracis. This three-protein toxin includes protective antigen (PA) and two enzymatic components, lethal factor (LF) and edema factor (EF), which must assemble into oligomeric complexes to disrupt cell physiology. Atx complexes are endocytosed, where they convert to a transmembrane channel that transports LF and EF into the cytosol. Assembly from monomeric components may occur in two physiological contexts: 1) in the bloodstream and 2) on cell surfaces. We have previously shown that the assembly of toxin complexes on cell surfaces produces a mixture of ring-shaped homooctameric or homoheptameric PA oligomers in a 1:2 ratio, which assemble via dimeric PA intermediates. Here we investigate how Atx complexes assemble in bovine blood. We find that, under