mainly due to a lack of relevant assays available at acidic pH. We have developed a single-molecule assay using force spectroscopy, which allows us to detect the reduction of single disulfides. The assay uses an Atomic Force Microscope to extend individual substrate proteins containing buried disulfides, thereby exposing the bonds to the solvent. When a disulfide bond is broken through reduction, this reaction is detected as a stepwise extension of the substrate polypeptide. This method enables measurement of reduction rates at a wide range of pH conditions. By altering the conformation of the disulfide through the applied strain on the substrate, we can dissect the mechanisms of enzymatic catalysis. Our results show that the enzymatic activity of GILT decreases as the strain on the substrate is increased. This feature is also seen in the enzyme thioredoxin but not in non-enzymatic reducing agents such as glutathione. These results shed light on the catalytic mechanism of GILT and establish single molecule force spectroscopy as a useful tool for characterizing enzymatic properties.

2327-Pos

Anisotropic Mechanical Response of the Enzyme Guanylate Kinase Perturbed by the DNA Molecular Spring

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Protein molecules are semi-rigid objects with organized but fluctuating conformation. For Guanylate Kinase, which catalyzes phosphoryl transfer between ATP and GMP, a large conformational change upon substrate binding occurs which is essential for enzymatic activity. With a DNA molecular spring stretching the molecule in distinct ways, we demonstrate that the enzymatic functions of substrate binding and phosphoryl transfer can be separately controlled mechanically.

Three different attachment points of the DNA spring on the surface of the protein were tested, corresponding to stretching the protein along three different directions. Using activity measurements with titration over substrate concentration, the kinetic parameters (i.e., binding affinity of substrates and catalytic rate constant) based on Michaelis-Menten kinetics were obtained in the presence and absence of the three different mechanical perturbations.

2328-Pos

Crystal Structure and Functional Analysis of Homocitrate Synthase, an Essential Enzyme in Lysine Biosynthesis

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Homocitrate synthase (HCS) catalyzes the first and committed step in the α-aminoadipate (AAA) pathway of lysine biosynthesis, which occurs in many fungi and certain archaea, and is a potential target for antifungal drugs. Here we report the crystal structure of the HCS apoenzyme from *Schizosaccharomyces pombe* and two distinct structures of the enzyme in complex with the substrate 2-oxoglutarate (2-OG). The structures reveal that HCS forms an intertwined homodimer stabilized by domain-swapping between the N- and C-terminal domains of each monomer. The N-terminal catalytic domain is comprised of a TIM barrel fold in which 2-OG binds via hydrogen bonds and coordination to the active site divalent metal ion, whereas the C-terminal domain is composed of mixed α/β-topology. In the structures of the HCS apoenzyme and one of the 2-OG binary complexes, a lid motif from the C-terminal domain covers the entrance to the active site of the neighboring monomer, whereas in the second 2-OG complex, the lid is disordered, suggesting that it regulates substrate access to the active site through its apparent flexibility. Steady state kinetic assays and *in vivo* yeast growth assays on wild-type enzyme and active site mutants allow us to elucidate its catalytic mechanism, including the residues implicated in catalysis. Together these results yield new insights into the mechanism and regulation of HCS, which provide a platform to identify small molecule inhibitors of HCS that may be optimized and used as anti-fungal agents.

2329-Pos

Kinetic Consequences of Mutations at an Allosteric Site in Arginase from the Thermophile *Bacillus caldovelox*

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Arginase is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea. It is involved in ureagenesis and the control of arginine levels for production of proline, creatine, polyamines, and nitric oxide. The crystal structure of the enzyme from the extreme thermophile *Bacillus caldovelox* reveal a second arginine-binding site, located at the monomer-monomer interface. Binding of the guanidinum group of L-arginine by bidentate hydrogen bonds to Glu256 in one monomer and bifurcated hydrogen bond to Asp199 in the neighbor, was suggested to generate a catalytically competent conformation. Interestingly, in the rat and human liver arginases, an equivalent position is occupied by Arg-308, which is part of an S-shaped C-terminal motif, that is critical for oligomerization and cooperative response to the substrate. The bacterial arginase lacks this motif. To get some insight into the external site in *B. caldovelox* arginase, we examined the kinetic and structural consequences of mutations at Asp199 to asparagine and Glu256 to glutamine. Upon mutations, the hexameric subunit structure, affinity of the enzyme-manganese interaction, pH and temperature dependence of catalytic activity, thermal stability and tryptophan fluorescence properties of the enzyme were not altered. However, the hyperbolic kinetics exhibited by the wild-type enzyme (K_m= 3.5 mM) changed to cooperative for both variants (Hill coefficients of 1.5 ± 0.2). Results were not altered by agmatine (decarboxylated arginine) or low concentrations of guanidinium chloride. Our conclusion is that occupancy of the second site by L-arginine is not required for generation of a catalytically competent active site. Instead, by binding at the allosteric site, L-arginine acts as a typical allosteric activator. Thus, the intrinsic cooperative behavior is exhibited by the mutants because of their inabilities to bind the allosteric activator. Fondecyt 1070467.

2330-Pos

Catalysis Mechanism of Aminopeptidase from Streptomyces Griseus: A Quantum Mechanical/Molecular Mechanical Analysis

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Aminopeptidases are exopeptidases that catalyze the removal of N-terminal amino acids for peptides [1]. X-ray revealed that the streptomyces griesus aminopeptidase (SGAP) is a double zinc proteolytic enzyme [2,3] with strong preference for large hydrophobic amino acids. Two different schemes for the general catalytic pathway of SGAP are proposed [1,4]: OH- or H2O nucleophilic attack mechanism. We are investigating SGAP's catalytic mechanism by means of hybrid quantum mechanical/molecular mechanical calculations (AM1d/MM) and analogous small molecule module mechanism with both AM1/d and B3LYP/6-31++G(d,p) methods. A complex network of reaction pathways is generated so as to explore a variety of different putative reaction mechanisms. Our molecular dynamics simulations (SGAP binded with MET-ALA-ALA) for different protonation pattern in the active site indicate that the most probable scenarion is a nucleophilic attack by a Zn2+-bound hydroxide ion, with the GLU131 protonated. Small molecular model AM1 calculation with Gaussian 03 indicates that the Zn2+-bound hydroxide ion first attack the backbone C(O) of MET and then H of this hydroxide ion "migrate" to the adjacent N of ALA, then the C(O)-N peptide chain between MET and ALA is cleaved.

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2331-Pos

Substrate-Induced *Eisenia fetida* Protease Reactions Involve Both "Induced Fit" and "Lock and Key" Mechanisms

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The coupling between ligand binding and protein conformational change is the heart of biological network. "Lock and key" theory and "induced fit" theory were early contributions to our understanding for explaining how an enzyme binds to a substrate. It was accepted that the binding of a substrate to an enzyme is often accompanied by conformational changes of the enzyme. However, whether the substrate-induced complementary conformation is flexible or rigid after a catalytic reaction remains to be determined. By testing the enzyme activity and intrinsic fluorescence of a substrate non-specific *Eisenia fetida* protease-I with different substrates, we show that when this enzyme reacts with a first substrate, it utilises the "induced fit" mechanism. However, in its reaction with further substrates, either the "lock and key" or "induced fit" mechanisms will be used depending on the degree of conformational change required. In contrast to the high activity of the native protease, the chromozym-Th (or -Ch)-induced protease was unable to react with chromozym-U. Chromozym-U-induced enzyme, however, had high activity with chromozym-Th and chromozym-Ch. When low concentrations of GuHCl were used to disturb the conformation of the enzyme, only small changes in intrinsic fluorescence of the CTH-induced protease were

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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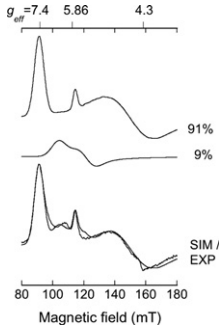
Lipoxygenases employ a redox-active metal center (Fe or Mn) in electron-proton coupled reaction to initiate oxidation of unsaturated fatty acids. In this study, the iron center geometry is examined by EPR for two newly characterized bacterial lipoxygenases, and the data are compared with similar studies 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,