was found to be an inhibitor of the enzyme subsequent to the crystallization. However at present no evidence suggests that the inactivation is due to the oxidation of the active site cysteine sulfenic acid.
The structures give insight into the determinants of the specificity of the enzyme, suggesting that βphe55 and βphe55 obstruct access to the cysteine claw complex for larger substrates allowing the hydration of only smaller aliphatic nitriles.

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Crystal Structure Of Peroxide-bound Manganese Superoxide Dismutase
Gloria Borgstahl, Jason Porta.
University of Nebraska Medical Center, Omaha, NE, USA.
The superoxide dismutase (SOD) enzymes are important antioxidant agents that protect the cells from reactive oxygen species (ROS). The SOD family is responsible for catalyzing the disproportionation of superoxide to oxygen and hydrogen peroxide. We report the first ever structure of a superoxide dismutase with bound hydrogen peroxide in the active site. Synchrotron X-ray diffraction data was collected from *Escherichia Coli* MnSOD crystals that were soaked in a cryosolution containing 0.008%(v/v) hydrogen peroxide and cryocooled to 100K. Structural refinement to 1.55 Å and close inspection of the active site revealed electron density for hydrogen peroxide in three of the four active sites. The hydrogen peroxide molecules are sideways bonded to the manganese in the position normally assumed by water or inhibitory hydroxide. The hydrogen peroxide molecules are present in active sites B, C and D. It was observed that MnSOD enzymes could accommodate two hydrogen peroxide molecules per active site in an antiprismo coordination geometry. Comparison of the peroxide-bound active site with the wild-type trigonal bipyramidal form shows a shifting of the gateway residues Tyr34 and His30, thereby preventing the escape of the bound ligands. The peroxide-bound form more closely resembles the active-site geometry of six-coordinate octahedral form (1D5N), where hydroxide ligands were trapped in the active site by cryocooling.

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Characterization of the Monomer-Dimer Equilibrium of Recombinant Histo-aspartic Protease from Plasmodium falciparum
Huogen Xiao[1], Lee-Ann Briere[2], Stanley D. Dunn[2], Rickey Y. Yada[1].
[1]University of Guelph, Guelph, ON, Canada, [2]University of Western Ontario, London, ON, Canada.
Malaria is a devastating disease that infects and kills 1-2 million people annually. Histo-aspartic protease (HAP) from *Plasmodium falciparum*, the most lethal of all Plasmodium parasites, is an intriguing aspartic protease due to its unique structure and its potential as an antimalarial target. Substantial effort has been devoted to investigate the structure function of this protease. The present study investigated the molecular state of HAP as related to enzymatic activity. Gel filtration chromatography indicated that recombinant Trx-tHAP fusion protein aggregated during purification and that aggregation could be prevented through the addition of 0.2% CHAPS. Using this latter technique as well as sedimentation velocity and sedimentation equilibrium ultracentrifugation, it was shown that the recombinant mature HAP (mtHAP), in which the His-tag, thioredoxin and prosegement were removed, exists in a dynamic monomer-dimer equilibrium in solution and the dissociation constant is 20-30 μM. Enzymatic activity data also indicated that HAP was most active as a monomer. The monomeric form of mtHAP showed a Km of 9.7 μM and a turnover number, kcat, of 0.044s-1 on the internally quenched fluorescent synthetic peptide substrate EDANS-CO-CH2-CH2-CO-Ala-Leu-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro-Dap-(DABCYL)-OH (2837b) at pH 6.5. Inhibition studies showed that the activity of mtHAP was completely inhibited by 1 mM PMSF and to a lesser degree by 10 μM ALLN, 10 mM EDTA and 10 mM 1,10-phenanthroline, and was inhibited strongly by ZnCl2 and to a lesser extent by NaCl and KBr. The effects of temperature and salts on the monomer-dimer equilibrium of mtHAP were also investigated by using sedimentation equilibrium ultracentrifugation.

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Analysis of Monomeric and Dimeric Phosphorylated Forms of PKR
Christine Quartararo, Eric Anderson, Raymond Brown, Yu Shi, Xudong Yao, James Cole.
University of Connecticut, Storrs, CT, USA.
PKR (protein kinase R) is induced by interferon and is a key component of the innate immunity antiviral pathway. Upon binding dsRNA or dimerization in the absence of dsRNA, PKR undergoes autophosphorylation at multiple serines and threonines that activate the kinase. Although phosphorylation is known to enhance PKR dimerization, gel filtration analysis reveals a second, monomeric phosphorylated form. The monomeric and dimeric forms do not interconvert. The monomeric form dimerizes weakly with a Kd similar to unphosphorylated PKR. Isoelectric focusing and mass spectroscopy reveal that both the monomeric and dimeric forms are heterogeneous in their phosphorylation state. Equilibrium chemical denaturation analysis indicates that phosphorylation destabilizes the kinase domain by about 1.5 kcal/mol in the dimeric form but not in the monomeric form. Limited proteolysis also reveals that phosphorylation induces a conformational change in the dimeric form that is not detected in the monomeric fraction. The monomeric phosphorylated form binds dsRNA similarly to unphosphorylated PKR but the affinity is greatly reduced for the dimeric form. Despite these differences in biophysical properties, both phosphorylated forms are catalytically competent and are activated to phosphorylate the PKR substrate eIF2a in the absence of dsRNA. Thus, both monomeric and dimeric forms of phosphorylated PKR may participate in the interferon antiviral pathway.

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Structural Studies of Enzymatic Hydrolysis of Cellulose by Neutron Scattering and Reflectivity
Michael S. Kent[1], Jaclyn K. Murton[1], Elizabeth L. Carles[1], Frank Zendejas[2], Rex Hjelm[3], Bulent Akgun[4], James Browning[5], John Ankner[5], Jacob Urquidi[6], Blake Simmons[2].
[1]Sandia National Labs, Albuquerque, NM, USA, [2]Sandia National Labs, Livermore, CA, USA, [3]Los Alamos National Labs, Los Alamos, NM, USA, [4]NIST, Gaithersburg, MD, USA, [5]Oak Ridge National Labs, Oak Ridge, TN, USA, [6]New Mexico State University, Las Cruces, NM, USA.
Improving the efficiency of enzymatic hydrolysis of cellulose is a key technological hurdle in reducing the cost of producing ethanol from lignocellulosic material. Typically, enzymatic hydrolysis proceeds to only a limited extent, high solution-to-solids ratios are required, and the rate of enzymatic hydrolysis typically decreases with time. A range of mechanisms have been proposed to explain these phenomena including product inhibition, denaturation of enzymes, nonproductive binding, and many others. We are studying the interaction of enzymes with cellulose to help unravel these mechanisms. Our studies include UV absorption and circular dichroism of enzymes in solution, small angle neutron and X-ray scattering (SANS, SAXS) of cellulose during hydrolysis, and neutron reflectivity (NR) of enzymes interacting with model cellulose surfaces. Insight from these studies should aid the development of more efficient enzyme systems and pretreatments.

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Expression And Purification Of A Stable, Monomeric Creatine Kinase
Gregg G. Hoffman, W. Ross Ellington.
Florida State University, Tallahassee, FL, USA.
Three isoform gene families of creatine kinase (CK) are present in animals. Two of these, mitochondrial and cytoplasmic CKs, are obligate oligomers . There is substantial evidence for functional interaction between subunits. Attempts at generating active, monomeric CKs have failed or in one case produced ephemerally active but unstable monomers. A third CK isoform, the so-called flagellar CK, is monomeric but is composed of three complete, contiguous CK domains. Each of these domains is catalytically competent but there is clear interaction between active sites (Hoffman et al., FEBS J 275: 646-654 [2008]). In the present effort, we have used a flagellar CK expression construct as a platform to engineer, express and purify a single domain, monomeric CK. Boundaries between the three domains (D1, D2 and D3) were identified by comparison of key catalytic residues and predicted secondary structural elements. A cDNA coding for D2 was amplified by PCR and inserted into an expression vector. Subsequent expression and purification yielded a recombinant CK which was stable as evidenced by the retention of activity over several weeks. Size exclusion chromatography showed that this CK was monomeric as expected, with a mass similar to the predicted Mr based on the amino acid composition. The engineering of a monomeric CK in the present effort clearly shows that oligomerization is not required for catalysis. Conventional wisdom supports the view that CKs evolved from a related phosphotransferase, arginine kinase (AK). AKs are typically monomeric. It seems likely that oligomerization occurred later in the evolution of CKs perhaps due to the selective pressure for targeting to and binding in intracellular compartments. (Supported by NSF grant IOB-0542236 to WRE).

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Role of Rim Tyr/Trp Residues in Interfacial Activation of Phospholipase C Enzymes
Su Guo, Mary F. Roberts.
Boston College, Chestnut Hill, MA, USA.
The *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (btPI-PLC) as a model system and mammalian PI-PLCδ1 without an intact PH domain have been examined for the contribution of rim aromatic groups to protein binding to vesicles and the correlation of this with catalytic activity. In the bacterial enzyme, two tryptophan residues (Trp47 in the two-turn helix B and Trp242 in a disordered loop) are critical for binding to interfaces; of the many several Tyr residues mutated, replacement with alanine at several sites (close to helix B as well as the active site) weakens membrane binding. For many of these residues the loss in binding affinity approximates what is

predicted for an Ala for Trp (or Tyr) substitution. If a single Tyr is modified there is little effect on the catalytic activity measured with mM substrate (although for Y88A an increase in specific activity is seen). In the mammalian enzyme, one Trp is in the hydrophobic rim ridge and could be analogous to Trp47 in bacterial PI-PLC. Replacement of the rim Trp has little effect on binding of the protein to non-substrate containing vesicles (measured by RET of the protein to labeled PE incorporated into the vesicles). However, the activity is significantly reduced. These results (and analyses of other surface variants) are discussed in terms of the multi-domain structure of the mammalian PLC contributing to binding but with X-Y domain exhibiting similar conformational changes to the bacterial enzyme.

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Association Between Enzymes Modifies the Inhibition by Trehalose

Daniela Araiza Olivera Toro[1,2], Antonio Peña Díaz[1,2], Salvador Uribe Carvajal[1,2].
[1]Universidad Nacional Autonoma de México, Mexico, Mexico, [2]Institute of Cell Physiology, Mexico, Mexico.

The crowding of the cell restricts the diffusion of solutes, provides specific binding sites for enzymes and promotes proteic interactions, allowing the metabolic channelling which favours a series of reactions in a pathway. (Srere PA. *Annu Rev Biochem*. 1987; 56:89-124) This process needs stable enzymatic interactions with low diffusion rate that give rise to multienzymatic complexes named metabolon.

The association modifies the kinetic properties and the relation between products and substrates, setting out that the enzymes form a complex among them or cellular structures. This suggests that the structural enzymatic organization exerts some control on the cellular metabolism.

Some factors can modify the equilibrium between the associated and soluble proteins, one of them being the viscosity promoted by the excess of compatible solutes like trehalose. (Kaushik J &, Bhat R. *J Biol Chem*. 2003; 278(29): 26458-65).

We decided to evaluate the effect of the trehalose over the glycolisis in yeast *Saccharomyces cerevisiae.* We had observed that in citoplasmic extracts the glycolisis is almost not affected by trehalose. But when we analyze some isolated enzymes we detected that some enzymes as aldolase and phosphoglycerate kinase (PGK) are not inhibited, while others like hexokinase and glyceraldehyde 3-phosphate dehydrogenase (GA3PDH) are inhibited. Enzymes of other pathways like glucose 6-phosphatase and glucose 6-phosphate dehydrogenase are also inhibited by the disaccharide.

To explain our results we did experiments with one sensible enzyme such as GA3PDH and a resistant PGK. This two may be associated and be part of the glycolitic metabolon. So in the coupled assay, the GA3PDH exhibit resistance to inhibition by trehalose. This suggests that the association stabilizes the sensible enzyme and it is probably specific because the combination of the GA3PDH with albumin, hexokinase and lactate dehydrogenase does not increase the resistance to trehalose.

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Kinetic Activity of the Intact 26S Proteasome in Mice Liver: Selective Regulation of Estrogen on the Core ß2 Subunit

Yanan Liu, Yong Wu, Mansoureh Eghbali, Ligia Toro, Enrico Stefani.
UCLA School of Medicine, Los Angeles, CA, USA.

The 26S proteasome complex plays an essential role in intracellular protein degradation. The 26S complex contains one 20S proteolytic core and two 19S regulatory particles. We investigated the kinetic properties of the 26S proteasome and the potential regulation by estrogen in mice liver in control conditions (ovariectomized placebo treated) and after 10 days of estrogen treatment. Livers were homogenized with a low concentration of detergent to preserve proteasome integrity (mM): 50 Tris-HCl, 250 sucrose, 5 MgCl2, 2 ATP, 1 DTT, 0.5 EDTA, and 0.025% digitonin, pH 7.5. The assay buffer contained (mM): 50 Tris-HCl, 40 KCl, 5 MgCl2, 0.5 ATP, 1 DTT, pH 7.5. Activity was measured at 37 oC using three fluorescent substrates, ß1, caspase-like (Z-LLE-AMC), ß2, trypsin-like (Boc-LSTR-AMC), and ß5, chymotrypsin-like (Suc-LLVY-AMC). With all substrates, the proteolytic kinetics showed three phases: 1) a delay reflecting an initial rate-limiting process (binding of the peptide substrates to the 19S regulatory particles and the translocation to the proteolytic core), 2) a linear time-dependent proteolysis (degradation process in the 20S chamber), and 3) a saturation phase. Activity was measured as a function of substrate concentration (10-500μM) at constant total protein (ß1, ß2 100 μg; β5, 50 μg). Increasing the substrate concentration did not affect the delay phase, while it increased the degradation rate and the saturation level. Increasing the protein concentration with constant substrate ß1, ß2 and ß5 50 μM) seemed to reduce the delay phase, while the linear activity and the saturation levels peaked at 100 μg protein. Estrogen treatment selectively stimulated proteolytic activity of the ß2 subunit trypsin-like activity. We conclude that proteasome activity has at least three sequential states with selective modulation by hormones of the proteolytic activity.

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Characterization of the Calcium Binding Domain of NADPH Oxidase 5 (NOX5)

Chin-Chuan Wei, Liu Qi Chen, Tremylla Johnson.
Southern Illinois University Edwardsville, Edwardsville, IL, USA.

Superoxide generated by non-phagocyte NADPH oxidases (NOXs), such as NOX5, is of growing importance for vascular physiology and pathology. NOX5 enzyme consists of a transmembrane heme domain that is linked to a flavoprotein domain that contains FAD and binds NADPH. It appears to be regulated by self-contained Ca^{2+} binding domains (CaBD), which contains four EF-hands motifs. Previously we demonstrated that this calcium binding gates the heme reduction in NOX5, possibly thorough the CaBD-flavorprotein interaction. To better understand its structure and function, here we characterized the metal binding properties of the recombinant CaBD by fluorescent spectroscopy. Our data revealed that CaBD binds to Ca^{2+}, Mg^{2+}, terbium (Tb^{3+}) in the range of μM to mM. The data are further supported by the studies using the site-directed labeled CaBD. The rate of calcium association was too fast to be determined by a stopped-flow device, but the dissociation rate constant was determined to be 5 s^{-1} at 20 °C. The ANS titration and Stern-Volmer plots suggested that there was a significant conformational change upon the metal bindings. Interestingly, the spectra of circular dichorism indicated otherwise no change on the context of its secondary structure. However, this conformational change can be observed using the Surface Plasmon Resonance with the CaBD immobilized in the sensor chip. Because our data and other studies suggest there are two different types of calcium bindings in CaBD, currently we are performing the similar studies using N- and C-terminal halves of CaBD (aa 1-78 and 79-184), and mutants. We also are investigating the metal bindings in the CaBD of Dual Oxidase (DUOX), in which its hydrogen peroxide activity is controlled by calcium binding.

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Enzymatic Activity and Monolayer Binding of a Truncated Form of Lecithin Retinol Acyltransferase

Sylvain Bussieres, Jean-Sebastien Laliberte-Gemme, Rock Breton, Christian Salesse.
Universite Laval, Québec, QC, Canada.

Lecithin retinol acyltransferase (LRAT) is a 230 amino acids membrane-associated protein. It has two enzymatic activities: first, it catalyzes hydrolysis of the sn-1 acyl chain of phospholipids and then transfers this acyl group to all-trans retinol to generate all-trans retinyl esters. This reaction is essential in the vertebrate visual cycle. The present study was performed to study the enzymatic activity of a truncated form of LRAT (tLRAT), where transmembrane domains have been removed. tLRAT extends from residues 31 to 196. It has been previously determined that the deleted domains of tLRAT do not contain residues known to be required for catalysis. tLRAT has been produced in *E. coli* and purified using affinity chromatography. Its enzymatic activity was studied using the short-chain diheptanoyl phosphatidylcholine (DHPC), which behaves like a mild detergent. The low critical micellar concentrations of DHPC allows to solubilize tLRAT and retinol. The maximal enzymatic activity of tLRAT is approximately 900 mol of ester/min • mol of protein. This value is more than 20 000 times higher than the largest enzyme activity reported in the literature. This huge difference can be explained by the use of a solution where DHPC serves both as a substrate as well as to solubililize the second substrate which highly favors the hydrolytic activity of tLRAT. Moreover, the injection tLRAT into the subphase of a phospholipid monolayer at different initial surface pressures allowed to determine the maximum insertion pressure (MIP) of tLRAT. A similar MIP of 38 mN/m has been obtained for dioleoyl phosphatidylcholine, ethanolamine and serine which is much higher than the lateral pressure of membranes. It can thus be postulated that tLRAT strongly binds membranes in the absence of its putative N- and C-terminal transmembrane domains.

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The Completion of chemo-mechanical coupling scheme of F_1-atpase; The Determination of the timing of Pi-release

Rikiya Watanabe, Hiroshi Ueno, Hiroyuki Noji.
Institute of scientific and industrial research, Osaka university, Ibaraki, Japan.

F_1-ATPase ($\alpha_3\beta_3\gamma$) is a rotary motor protein, which makes 120° step rotation upon one ATP hydrolysis. Extensive studies on F_1-ATPase revealed that each of three β-subunits, which has the catalytic site, follows the same reaction pathway of ATP hydrolysis, but they are always in a reaction phase differing by ±120° from each other. When we focus on one β-subunit, the β binds ATP at a particular binding angle. After the γ rotates 200°, the β cleavages the bound ATP into ADP and Pi. The produced ADP is released from the β after further 40° rotation, at +240° from the ATP-binding angle. Then, when the γ makes