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

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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

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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)

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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

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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

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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

one revolution, the β returns to the ATP waiting state again; however, it has not been identified where the bound Pi is released, although it was suggested to occur at +200° or +320° from the ATP-binding angle. In this study, we observed the rotations of the hybrid F_1-ATPase, $\alpha_3\beta(WT)_2\beta(E190D)_1\gamma$ with the high-speed camera. At +320° from the ATP binding angle of the incorporated mutant β(E190D), the clear pause of ~7msec was observed as reported previously (Ariga et al. Nat. Struct. Mol. Biol.). When high concentrations of Pi was added to the solution, the time constant of the new reaction was specifically prolonged upon addition of Pi, suggesting that Pi was released at +320°. Other lines of experiments also support this result.

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PTEN Inhibition Study by Synthetic 3-Deoxy-PI Derivatives

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PTEN is a tumor suppressor mutated in a large variety of human tumor cells. It antagonizes the PI3K signaling pathway by dephosphorylating the $PI(3,4,5)P_3$ at the 3 position of the inositol ring and plays an important role in cell growth, proliferation, survival and motility. 3-Deoxy-PI derivatives have cytotoxic activity against various human cancer cell lines by a mechanism thought to involve reduced Akt1 phosphorylation. However, these molecules could act as inhibitors of PTEN. A series of D-3-deoxy-PI derivatives and their enantiomers (3-deoxy-diC_8PI, 3,5-dideoxy-diC_8PI and diC_8PI) had been synthesized and studied as inhibitors of PTEN catalyzed hydrolysis of PI(3)P substrates. With diC_8PI(3)P as the substrate, the L- enantiomers are usually not as good inhibitors as the D-compounds, although there is an increase in potency with increasing deoxygenation. The short-chain lipids exist in the assay solution as a mix of mostly monomers but with some small micelles. By using D-diC_{16}PI(3)P as the substrates, the effect of the aggregation state of the substrates was also checked. When the substrates are presented to PTEN in micelles with TX-100, none of the 3-deoxy-PI derivatives are good inhibitors. However, when the substrates are presented in large unilamellar vesicles, the inhibitory behavior of the 3-deoxy-PI derivatives is similar to what was observed in the diC_8PI(3)P monomer/micelle system. The binding behavior of PTEN to PI vesicles in the presence of the deoxy-PI analogs has also been studied using FRET. The results showed that for the L-series, deoxygenation on the inositol head group increases the potency of enhancing the protein binding to the vesicles. In this case, L-3,5-dideoxy-diC_8PI can enhance binding by 50% at the lower concentration, while L-diC_8PI can only enhance binding at the high concentration larger than 1mM. These results are used to assess lipid binding sites in PTEN.

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The role of Kallikrein-kinin System in the Immune Response of Nasal Papilloma and Adventitious Sinusitis

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The work was dedicated to study the possible interconnection between the enzymatic activity of kallikrein-kinin system and the activation of immune response of benign nasal papilloma and adventitious sinusitis. 64 patients with nasal papilloma and adventitious sinusitis have been examined for the enzymatic activity of kallikrein-kinin system and for the cellular and humoral immune responses, as well as 20 healthy. The enzymatic activity of kallikrein-kinin system and antibody response were investigated by using the sera or plasma samples. Cellular immune response was evaluated by analyzing of T- lymphocytes, B- lymphocytes and O- lymphocytes. Comparative analysis of kallikrein activity and cellular immune response showed that elevation of kallikrein activity was well correlated with activation of T- lymphocytes (r=0.896) and T-supressors (r=0.975), indicating that the activity of kallikrein may be important in modulation of T-cell response. In contrast, activation of kallikrein-kinin system was not associated with induction of B-lymphocytes (r=0.578), T-helpers (r=0.694) and O-lymphocytes (r=0.569). Analysis of kallikrein activity and humoral immune response in the same study group showed that induction of kallikrein-kinin system was associated with substantial elevation of IgA (r=0.785), in contrast to lower level of IgG and IgM expression.
It is also revealed that there is a high correlation between kininase activity and the indices of B-lymphocytes (r=0.768), T-supressors (r=0.754) and concentration of IgA (r=0.889), IgG (r=0.995) and IgM (r=0.889), in it the kininase ferment plays an important part for the formation of IgG.
Our data suggested that the kallikrein-kinin system may play a regulatory role in cellular and humoral immune response and such interconnection between these two systems could be used as additional criteria for the evaluation of immune status.

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Induction Of Functional Hypoxia-inducible Factor-1α And Angiogenesis By Derivatives Of 8-hydroxyquinoline

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Hypoxia-inducible factor-1 (HIF-1) as a complex of α and β subunits mediates a ubiquitous pathway by which mammalian cells sense and respond to hypoxia. In mammalian cells, the levels and activity of HIF-1α are regulated by its post-translational hydroxylation as catalyzed HIF hydroxylases, whose inhibition is thus attractive from the perspective of developing pharmaceuticals that activate the HIF pathway and induce a pro-angiogenic response. We found that 8-hydroxyquinoline and its derivatives inhibit hydroxylation of proline and asparagine of HIF-1α with varying degrees. In addition, they completely block ubiquitination of HIF-1α, which leads to its accumulation and activation of HIF-1-mediated vascular endothelial growth factor transcription and reporter gene activity. Furthermore, in vivo organ models based on the chick chorioallantoic membrane assay demonstrate promotion of new blood vessel formation. Therefore, our results indicate that CQ analogs possess a pro-angiogenesis potential and thus might have the therapeutic utility in the treatment of ischemic diseases.

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The Exit of the Tunnel: Yeast Alcohol Dehydrogenase from the Acceptor's Point of View

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Alcohol dehydrogenase (ADH) is a popular model used to study quantum mechanical phenomena in enzyme-catalyzed reactions. Studies of α-secondary kinetic isotope effects (2° KIEs) have shown that the oxidation of benzyl alcohol by NAD^+ occurs by quantum tunneling with coupled motion of the primary and secondary hydrogens. In order to learn more about the nature of that coupling, we have measured α-secondary KIEs in the reverse reaction, i.e., for the yeast ADH (yADH) catalyzed reduction of benzaldehyde to benzyl alcohol. Preliminary results show that whether ^1H or ^2H is being transferred, the reaction maintains normal 2° KIEs ($k_H/k_T > 1$). This is most significant given that the equilibrium isotope effect for this process is inverse (EIE = 0.75). Semi-classical theory predicts an inverse 2° KIE ($k_H/k_T < 1$) for this reaction, thus the findings support a role for quantum mechanical H-tunneling in the reduction of aldehydes by yADH. Furthermore, these 2° KIEs violate the rule of the geometric mean: the semi-classical formulation of KIEs that predicts no difference in 2° KIEs upon isotopic substitution at the primary position. In this reaction, however, the magnitude of the 2° KIE decreases significantly when the transferred isotope is ^2H. Together, these results provide strong support for the model of tunneling and 1°-2° coupled motion used to describe enzymatic H-transfers.

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Mechanism of action of cyclophilin A explored by metadynamics simulations

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Peptidyl prolyl isomerases (PPiases) are ubiquitous enzymes that catalyze the interconversion of the *cis* and *trans* isomers of the peptidyl prolyl bond. Their action is crucial in several biological processes as, for instance, in cellular signalling and in the onset of several diseases. In the HIV-1 capside protein (CA), such process takes place in the uncoating and recruitment of the virion and is catalyzed by cyclophilin A (CypA). Previous studies identified several residues that play an important role in the *trans/cis* interconversion process. However, the role of some active site residues remains still obscure, since their catalytic importance depend crucially on the stabilization of both ground and transition states. Here, we report the results of classical AMBER99 calculations on a substrate fragment of the capside protein (the /HAGPIA/ peptide) in aqueous solution and in complex with CypA. By applying replica exchange metadynamics, we calculate the free energy profile of the isomerization process in both cases as a function of several reaction coordinates. We find that CypA catalyzes only one isomerization pathway in the *trans*-to-*cis* direction and enhances the stability of a particular *cis* conformer. Based on our computational results, we propose a novel hypothesis for the working mechanism of cyclophilin A that explains, for the first time, all the available data and awaits further experimental tests.