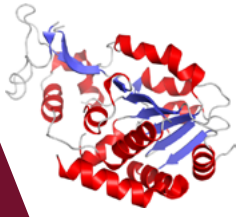
PROTEINE 2018

VERONA



Silos di Ponente del Polo di Santa Marta · University of Verona

MAY 28 - 30, 2018

ABSTRACT BOOK

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SCIENTIFIC PROGRAM

May 28, 2018

14:00 Participants Registration

Session I · Protein Interactions and Dynamics

Chairs: Rita Casadio, M. Eugenia Schininà

15:00 Annalisa Pastore, *The Francis Crick Institute, London (UK)*
The intricate network of interactions in iron-sulfur cluster biogenesis

15:40 Margherita Ruoppolo, *Università degli Studi di Napoli Federico II*
Altered intracellular pathways in inherited disorders of vitamin B12 metabolism

16:10 Luca Federici, *Università degli Studi Gabriele D'Annunzio, Chieti-Pescara*
Exploring nucleophosmin interactions for the treatment of acute myeloid leukemia

16:40 *Coffee break*

17:10 Pier Luigi Martelli, *Alma Mater Studiorum Università degli Studi di Bologna*
Predicting protein-protein interaction sites with computational approaches

17:40 Alessandra Astegno, *Università degli Studi di Verona*
Plant calmodulin-like proteins: calcium binding and target interactions

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Pathogenic variants of human Aromatic L-Amino Acid Decarboxylase: evidences of misfolding in functionally active variants

Emanuela Leonardi, *Padova*
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19:00 *Welcome Cocktail at Santa Marta*

May 29, 2018

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SCIENTIFIC PROGRAM

10:30 Fabrizio Chiti, *Università degli Studi di Firenze*
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11:00 Alessandra Corazza, *Università degli Studi di Udine*
An NMR approach to structure and dynamics of human transthyretin

11:30 Damiano Piovesan, *Università degli Studi di Padova*
Computational resources for the study of intrinsically disordered proteins

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Stefania Brocca, *Milano*
How to design an (in)soluble protein tag: some insights from synthetic intrinsically disordered proteins

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Session III · Biocatalysis: mechanisms and applications

Chairs: Marco Moracci, Loredano Pollegioni

15:00 Gideon J. Davies, *University of York (UK)*
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15:40 Gianfranco Gilardi, *Università degli Studi di Torino*
Exploitation of monooxygenases in biocatalysis

16:10 Mario Cappiello, *Università di Pisa*
Intra-site differential inhibition of enzymes: the case of aldose reductase

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17:10 Andrea Strazzulli, *Università degli Studi di Napoli Federico II*
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Elena Rosini, *Varese*
Lignin valorization: the Lig system

Francesco Marchesani, *Parma*
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18:10 *Andrea Pigozzo, ALFATEST, Roma*
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SCIENTIFIC PROGRAM

May 30, 2018

8:30 Registration Desk opening

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9:00 Alessandro Vannini, *The Institute of Cancer Research, London (UK)*
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9:40 Marco Nardini, *Università degli Studi di Milano*
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10:30 Riccardo Miggiano, *Università degli Studi del Piemonte Orientale, Novara*
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11:00 Silvia Onesti, *ELETTRA Sincrotrone Trieste*
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Samanta Raboni, *Parma*
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Session I · Protein Interactions and Dynamics

S01

Plant calmodulin-like proteins: calcium binding and target interactions

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Calcium (Ca^{2+}) ions play a key role in a wide variety of environmental responses and developmental processes in plants, and a unique toolkit of proteins that bind Ca^{2+} using the evolutionarily conserved EF-hand motif have evolved to meet these needs. In addition to calmodulin (CaM), which has been well conserved through evolution, plant genomes are predicted to encode a broad range of calmodulin-like (CML) proteins (50 members in Arabidopsis[1]), which have no catalytic activity, but rather act as sensor relays that regulate downstream targets to propagate the information in signaling pathways.
Various approaches aimed at understanding the CMLs function are beginning to reveal that these proteins are not likely to have redundant functions, but rather play central and highly specific roles in plant development as well as in response to biotic and abiotic stress.[2] Whereas increasing information is now available that links CMLs function with defined biological processes, knowledge is limited on (i) the metal-binding and structural properties of this plant-specific class of Ca^{2+} sensors, and (ii) the identification of specific CMLs targets and the characterization of the CML-target interactions.
Our current research focuses on both these aspects. In particular, we have been systematically studying the biochemical properties of *Arabidopsis* CMLs, including CML7, CML14, CML19, and CML36 using combined biochemical and biophysical approaches. Globally, these analyses have allowed significant steps forward in appreciating how such a vast array of CML proteins can coexist, without apparent redundancy, and how they make a distinct contribution to cellular signaling, while being different from CaM and other Ca^{2+} sensors.

References

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Session I · Protein Interactions and Dynamics

P01 - Oral selected

Pathogenic variants of human Aromatic L-Amino Acid Decarboxylase: evidences of misfolding in functionally active variants

Bisello G., Montioli R., Dindo M., Voltattorni C.B., Bertoldi M.

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Dopa decarboxylase (DDC), a homodimeric pyridoxal 5'-phosphate (PLP)-dependent enzyme, is responsible for the decarboxylation of L-Dopa and L-5-hydroxytryptophan to neurotransmitters dopamine and serotonin, respectively. Missense mutations of the *DDC* gene lead to a rare and often fatal form of inborn infantile Parkinsonism, known as AADC deficiency [1]. Up till now, it was shown that an incorrect apo→holo DDC transition is the reason of the pathogenicity of the majority of the mutations so far examined, with the exception of the R347 mutations defective in catalysis [2]. Here we extend our work to a group of pathogenic mutations concerning residues distributed in different regions of DDC: P47H on the N-terminus, E283A, R285A, P210L and W267R on the large domain and R412W on the small C-terminal domain. The variants in their purified recombinant form exhibit k_{cat} and K_m values for L-Dopa as well as equilibrium binding dissociation constant values for PLP not very different from those of the wild-type. Thus, their functional properties do not seem to be responsible for their pathogenicity. Nonetheless, CD and DLS experiments highlight that all of these variants display even at different extent (i) an alteration of their tertiary structure and of their surface exposure features, and (ii) a propensity to an electrostatic-driven aggregation, mainly in their apo form. These data, together with the fact that these variants are expressed in *E.coli* at a level less than 1% of the wild-type, strongly suggest that folding defects characterize the variants. Thus, their inability to achieve or maintain a fully functional conformation would be the reason of their pathogenicity. Our results expand the knowledge of the enzymatic phenotypes leading to AADC deficiency.

References

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2 Montioli R. (2014), A comprehensive picture of the mutations associated with aromatic amino acid decarboxylase deficiency: from molecular mechanisms to therapy implications, Human Molecular Genetics, 23 (20) 5429-5440.

Session I · Protein Interactions and Dynamics

P02

Conformational and functional effects of CaF_2 nanoparticles on GCAP1, a neuronal Ca^{2+}-sensor involved in retinal dystrophies

Borsatto A. [1], **Marino V.** [2,3], **Vocke F.** [4], **Koch K.W.** [4], **Dell'Orco D.** [3]

[1] University of Trento
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[3] Dept. of Neurosciences, Biomedicine and Movement Sciences, University of Verona
[4] Dept. of Neuroscience, Biochemistry Group, University of Oldenburg

Calcium (Ca^{2+}) sensor proteins are capable of changing conformation upon detection of variations in Ca^{2+}-concentration, thus allowing the regulation of different molecular targets involved in several cellular processes. Neuronal calcium sensors like Guanylate Cyclase Activating Protein 1 (GCAP1) have been associated with retinal dystrophies, thus highlighting their importance as target for new therapeutic approaches.
CaF_2-based nanoparticles (NP) are promising biocompatible tools for nanomedicine applications. The structure of the NP crystal lattice allows for specific interactions with Ca2+-binding proteins through their EF-hand cation binding motifs.
We investigated the interaction of 23 nm citrate-coated CaF2 NP with wild type (WT) GCAP1 and the cone dystrophy-associated variant D100E, which prevents the binding of Ca^{2+} to the highest affinity site, in order to assess the potential of these NP as protein carriers.
Circular dichroism and fluorescence spectroscopy showed that protein structure and Ca^{2+}-sensing capability are conserved for both variants upon interaction with the NP surface, although the interaction mode depends on the specific occupation of Ca^{2+}-binding sites. NP binding stabilizes the structure of the bound GCAP1 and occurs with nanomolar affinity, as probed by isothermal titration calorimetry.
Dynamic light scattering and surface plasmon resonance revealed a fully reversible binding compatible with physiologically relevant kinetics of protein release whereas biochemical assays indicated a residual capability for NP-dissociated GCAP1 to regulate the target retinal guanylate cyclase[1]. Our study constitutes a proof of concept that CaF_2 NP could be optimized to serve as biologically compatible carriers of high amounts of functional GCAP1 in photoreceptors affected by retinal dystrophies.

References

1 Marino V., Borsatto A., Vocke F., Koch K.W., Dell'Orco D. (2017) CaF_2 nanoparticles as surface carriers of GCAP1, a calcium sensor protein involved in retinal dystrophies. Nanoscale. Aug 17;9(32):11773-11784.

Session I · Protein Interactions and Dynamics

P04

MHC class II β from the icefish *Chionodraco hamatus*: molecular and functional characterization, and 3D structure

Buonocore F.[1], Scapigliati G.[1], Gerdol M.[2], Pallavicini A.[2], Cimmaruta R.[3], Facchiano A.[4]

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The major histocompatibility complex class I and II molecules (MHC I and MHC II) are key components of the adaptive immune system. These molecules are heterodimers formed by α and β subunits which bind self and non-self-peptides and present them to T-cells. Teleost fish present a peculiar organization of the MHC I and MHC II loci that reside in two different linkage group in contrast to tetrapods and cartilagineous fish. The number of functional copies of MHC II β genes is highly variable across different teleost species and also within the same species. Antarctic notothenioid fishes provide an interesting opportunity to study the immune responses in a peculiar environment as low temperatures are thought to play a significant role in influencing the immune system and to modulate MHC genetic diversity. In our study, we identified two MHC class II β sequences from a gill transcriptome of the Antarctic icefish *Chionodraco hamatus*. These sequences belong to the classical (ChhaDAB) and nonclassical (ChhaDBB) evolutionary lineages. ChhaDAB showed high level of expression in different tissues, like gills and head kidney, whereas ChhaDBB was found only at low levels in gills. Using a deep sequencing approach, we investigated the variability of MHC exon 2 and evidenced the presence of 162 different allelic variants from 54 icefish individuals in ChhaDAB, but very low molecular diversity for ChhaDBB. 3D Protein modelling of ChhaDAB has been performed by comparative modelling with the template structure of corresponding molecule from mouse, and it generated high quality results. This model gives us the opportunity to evaluate the potential role of the variants in relation to the function of the molecule as the regions that are variable in ChhaDAB corresponds in the template crystal structure to domains that interacts with both MHC II α chain and T-cell receptor.

Acknowledgements
Research was performed under the Italian Program of Antarctic Research (PNRA), granted projects PNRA16_00099 and PNRA16_00279.

Session I · Protein Interactions and Dynamics

P05

Effects of oligomerization on peroxisomal import and stability of human alanine:glyoxylate aminotransferase

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Peroxisomal proteins synthesized in the cytosol are targeted to peroxisomes through signals (PTS) exposed on the fully-folded protein. The impact of the oligomeric state on peroxisomal import is still debated[1]. Alanine:glyoxylate aminotransferase (AGT) is a pyridoxal 5'-phosphate (PLP)-enzyme whose deficit causes Primary Hyperoxaluria Type I (PH1)[2]. AGT is homodimeric, but we have engineered a mutant form stable as monomer [3]. We used monomeric and dimeric AGT as models to investigate the effect of oligomerization on the intracellular behaviour and import of peroxisomal matrix proteins. We found that monomerization (i) does not prevent peroxisomal import, but strongly reduces AGT intracellular stability and increases its aggregation propensity, (ii) partly holds the protein in the cytosol, where it is prone to aggregation/degradation and mitochondrial mistargeting. FRAP experiments showed that dimeric and monomeric AGT display a similar import rate in-vivo, indicating that the oligomeric state does not influence import kinetics. The treatment with pyridoxine of cells expressing monomeric AGT increases the amount of dimeric folded protein and promotes the correct import, demonstrating the chaperone role of PLP. Thus, the main factor influencing the fate of peroxisomal proteins is not oligomerization *per se*, but rather the kinetics and equilibrium of the folding process. Besides explaining the effects of PH1-causing mutations that destabilize the AGT dimer, this is the first study addressing the relationship between folding and targeting for a matrix peroxisomal protein.

References

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Session I · Protein Interactions and Dynamics

P06

New weapons against antimicrobial resistance: Targeting SOS response to recover bactericidal activity of antibiotics

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Drug Resistant Bacteria represent a global emergency, limiting the effective treatment of bacterial infections.The development of novel strategies fighting bacterial infections is strongly desirable. The SOS pathway has been recently validated as a key target for combating the evolution of antibiotic resistance. In bacteria the SOS response is orchestrated by two proteins: RecA, the sensor protein, and LexA, the regulator one. As a consequence of damage to the DNA, RecA monomers can form large nucleoprotein filaments on single stranded damaged DNA and promote self-cleavage of LexA, a repressor binding a palindromic sequence of 16 base pairs (lexA binding Box), thus inducing the expression of more than 40 genes involved in DNA repair and mutagenesis.
In an effort to identify new LexA inhibitors, starting form the available LexA crystal structures, a structure based virtual screening of a database of available chemicals was conducted, searching for potential inhibitors able to block proteolytic activity of LexA C-terminal domain. In parallel, a full gene-to-crystal structure pipeline of a sequence coding for recombinant LexA C-terminal domain have been optimized in order to characterize and screen the most promising hits. In parallel, new approaches for high-throughput *in vitro* screening of small compounds libraries have been developing in our laboratory. Such an approach has the potential to open up new strategies for reversing drug resistance by targeting the SOS response.

Session I · Protein Interactions and Dynamics

P07

Involvement of Apoptosis Inducing Factor variants in neurodegenerative mitochondriopathies

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The Apoptosis Inducing Factor (AIF) is a highly conserved mitochondrial FAD-dependent flavoprotein, known to play two opposite (vital and lethal) roles in eukaryotic cells: inside mitochondria, it is required for efficient oxidative phosphorylation, whereas when released from the organelle, it causes caspase-independent apoptosis[1]. AIF is able to form a tight, air-stable charge-transfer complex (CTC) upon NAD(H) binding, leading to protein dimerization. Thus AIF might act as a redox sensor in the mitochondrial intermembrane space (IMS). Moreover, AIF was recently discovered to interact with CHCHD4, an IMS protein that regulates oxidative folding of respiratory complexes' subunits[2].
About ten point mutations of the human AIF gene were found to cause rare neurodegenerative mitochondriopathies. To get more insights on AIF variants pathogenicity, we selected a set of mutations (G337E[3], D236G[4], G261S[5] and a still unpublished one), to investigate their effects on AIF molecular properties and their interaction with CHCHD4. To this aim, a combination of spectrophotometric techniques, MicroScale Thermophoresis (MST) and structural biology was used. AIF variants CTC stability was evaluated monitoring its reoxidation by O2. Interestingly, CTCs of G337E and G261S forms displayed a faster oxidation compared to wild-type AIF. Furthermore, the 3D structures of AIF-D236G CTC and of the unpublished variant both in oxidized and CTC states were obtained, showing that these amino acyl replacements don't induce large structural rearrangements. Thus we speculated that AIF mutations might impair the interaction with CHCHD4. MST data revealed that, for all AIF variants, CTC formation increased the affinity for CHCHD4, with a similar extent to wild-type AIF. Further analyses are needed to elucidate the effects of AIF mutations at the molecular level.

References

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2 E. Hangen *et al.* (2015), Interaction between AIF and CHCHD4 Regulates Respiratory Chain Biogenesis, *Mol Cell*, 58(6):1001-14.
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4 H. Mierzewska *et al.* (2016), Spondyloepimetaphyseal dysplasia with neurodegeneration associated with AIFM1 mutation - a novel phenotype of the mitochondrial disease, *Clin Genet*, 91(1):30-37.
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Session I · Protein Interactions and Dynamics

P08

Guanylate Cyclase Activating Protein 1 mutants associated with retinal dystrophy: a biochemical investigation

Dal Cortivo G.[1], Marino V.[1], Oppici E.[1], Bonì F.[2], Milani M.[2], Dell'Orco D.[1]

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Guanylate Cyclase Activating Protein 1 (GCAP1) is a Ca^{2+}-sensor protein involved in the regulation of the target enzyme Guanylate Cyclase (GC), an important modulator of the phototransduction cascade, which initiates the visual process. To date, fifteen missense mutations have been found to be associated with cone and cone-rod dystrophy (COD and CORD), degenerative retinal diseases characterized by progressive loss of central vision and defective color perception. In order to provide new insights into functional effects of GCAP1 mutations, a combination of biochemical and biophysical techniques was used. After the expression and purification of the recombinant human WT GCAP1 and three mutants (D100G, E155G and I143N) structural and functional analysis were performed by using circular dichroism (CD), polyacrylamide gel electrophoresis (PAGE, both in the presence and in the absence of SDS), dynamic light scattering (DLS), fluorescence and absorption spectroscopy and, finally, analytical size exclusion chromatography. Our data clearly show that both WT and COD/CORD-related human GCAP1 forms a constitutive dimer in reducing conditions and at each tested concentration (2-90 μM). From a structural point of view, CD spectra did not display significant differences in thermal stability, secondary or tertiary structures for WT and mutated GCAP1 variants; the only exception is D100G-GCAP1, for which a reduced Ca^{2+}-induced response was observed. The most apparent difference between WT and dystrophy-related GCAP1 mutants is the affinity for Ca^{2+}: except for I143N, which shows a WT-like affinity, D100G and E155G are characterized by lower apparent affinity. These results will constitute an important starting point for the future design of molecules capable to restore physiological conditions.

Session I · Protein Interactions and Dynamics

P09

Identification and characterization of molecular interactors of the Receptor for Advanced Glycation End products (hRAGE)

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The Receptor for Advanced Glycation End products (hRAGE) is a transmembrane glycoprotein that plays beneficial role in physiological conditions, but it is also involved in the propagation of inflammation and diabetes[1].
Advanced Glycation/Lipoxidation End products (AGEs/ALEs), derived from the non-enzymatic glycation/lipoxidation of proteins, are among RAGE ligands, but the requirements for RAGE-AGEs/ALEs interaction still remain unclear. Moreover, AGEs/ALEs detection in biological samples is difficult due to their heterogeneity and low abundance.
In order to enrich and detect AGEs/ALEs, we developed a pull-down assay employing RAGE ligand-binding domain (VC1) as a bait[2,3]. To set up the pull-down assay, a new recombinant form of VC1 was expressed in *Pichia pastoris*, a suitable host for the secretion of glycosylated/soluble VC1[4]. Glycan moieties and thermal stability of the recombinant protein were characterized.
Here we focused on ALEs-HSA generated *in vitro*. First, ALEs-HSA adducts were characterized by Mass Spectrometry (MS), then the species interacting with VC1 were detected by pull-down assay and SDS-PAGE. Adducts specifically enriched by VC1 were identified by high resolution MS analysis. Results indicate that ALEs characterized by the presence of cyclic moieties are preferential interactors of VC1. An interpretation of the contacts established by ALEs-HSA adducts with VC1 was provided by computational studies.
The identification of the moieties that characterize ALEs as RAGE interactors could lead to the development of antagonists able to reduce the effects of ALEs-RAGE interaction. Moreover, this pull-down assay will be useful to enrich ALEs/AGEs from biological samples.

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Session I · Protein Interactions and Dynamics

S02

Exploring nucleophosmin interactions for the treatment of acute myeloid leukemia

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Nucleophosmin (NPM1) is a nucleus-cytoplasm shuttling protein implicated in ribosome biogenesis, centrosome duplication, cell cycle control and response to stress stimuli. It is one of the main "hub" proteins in nucleoli and, as such, it interacts with both nucleic acids and a plethora of other proteins including the tumor suppressors p14arf and Fbw7γ, the main E3-ubiquitin ligase of c-MYC.
NPM1 is the most frequently altered protein in acute myeloid leukemia (AML), accounting for 30% of total patients and 60% of those with normal karyotype. Mutations hit the NPM1 C-terminal domain and lead to domain unfolding, impaired interactions with nucleic acids, loss of the nucleolar localization signal and the appearance of a novel nuclear export signal. As a consequence the protein is stably and aberrantly delocalized in the blasts' cytoplasm. While translocating in the cytoplasm, mutated NPM1 carries with itself a number of protein partners, including p14arf and Fbw7γ, which are then degraded. For this reason, NPM1 mutation results in the impairment of a critical p14arf-HDM2-p53 tumor suppressor axis and in c-MYC stabilization. In the last few years, we analyzed the interaction of NPM1 with p14arf and Fbw7γ at the molecular level by combining NMR, molecular dynamics, fluorescence spectroscopy and site-directed mutagenesis and identified critical residues in both NPM1 and protein partners. We also tested the possibility of interfering with NPM1 protein-protein interactions as a route for treating leukemia with NPM1 mutations. We report on the activities played by N6L, a positively charged pseudopeptide, on AML cell lines bearing NPM1 mutations or not, alone or in combination with conventional chemotherapy.

Session I · Protein Interactions and Dynamics

P10

Interactions with biological macromolecules of five-coordinate Pt(II) compounds containing sugar ligands

Ferraro G., Cucciolito M.A., D'Amora A., De Feo G., Giorgio A., Petruk G., Monti D.M., Ruffo F., Merlino A.

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Cytotoxicity of novel cationic five-coordinate Pt(II) compounds containing nitrogen sugar-based ligands has been evaluated on different cell lines with the expectation that both the coordinative saturation and the sugar moiety cooperate to enhance their biological activity. These complexes are more active than cisplatin but present little selectivity. Binding of representative compounds with DNA was studied by ethidium bromide displacement assay and circular dichroism. Binding to model proteins was investigated by UV-Vis absorption spectroscopy and X-ray crystallography. The structure of the adduct formed in the reaction between a representative compound and the model protein bovine pancreatic ribonuclease was refined at 1.14 Å resolution. Data indicate that the cytotoxic compound is able to bind the protein, with Pt center reacting with imidazole of the His side chains that are on the protein surface, upon releasing one of its ligand, but retaining the five-coordinate geometry. The overall structure of the protein is not affected by the compound binding.

References

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Session I · Protein Interactions and Dynamics

P11

Human angiogenin dimerization and S28N pathogenic mutation

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Human angiogenin (ANG), also called RNase 5, is a 123 aa residues protein which belongs to the "pancreatic-type" ribonuclease super-family[1] and is endowed with angiogenic and neuroprotective activities[2]. ANG folds similarly to the super-family proto-type RNase A, but its catalytic activity is $10^5/10^6$ fold lower because the active site is hindered by the Q117 residue. However, the ribonucleolytic activity is necessary for ANG physiological functions[2]. Considering its angiogenic function, ANG is also involved in tumorigenesis[1]. Moreover, missense mutations affecting the ANG gene coding region were found in patients affected by amyotrophic lateral sclerosis (ALS) or Parkinson's disease (PD)[3]. These mutations induce ANG loss of function and a decrease of the neuroprotective activity of the wild-type[4]. Also a possible ANG self-association and precipitation in CNS may be a consequence of the pathogenic mutations.
We produced the pathogenic S28N-ANG variant that was reported to spontaneously form traces of a not well characterized dimer[5], in addition with loss of both enzymatic and angiogenic activities. We observed the presence of a dimer during S28N expression and we found that both wild type and S28N-ANG form more than 10% dimer after acid lyophilisation performed as for RNase A[6]. We are currently investigating if the latter dimer forms upon the 3D domain swapping mechanism[6], while other structural and functional investigations on both dimers are under way.

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Session I · Protein Interactions and Dynamics

P12

Calmodulin and calmodulin-like protein 36: diverse binding modes to a common target?

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In addition to the well-known Ca^{2+} sensor calmodulin (CaM), plants possess many CaM-like (CML) proteins, which are emerging as central and highly specific effectors in coordinating plant environmental responses. Up to now, several CML targets have been identified by protein microarray analysis, in addition to genetic and in vivo studies. These investigations have assigned relatively specific physiological roles to several CMLs, different from CaM, which has broad target specificity. Nevertheless, the existence of overlapping targets between CMLs and CaM has been suggested.
Recently, CML36 was found to interact with the regulative N-terminus of the Arabidopsis plasma membrane Ca^{2+}-ATPase isoform 8 (ACA8), stimulating its activity[1]. The best known regulator of ACA8 is CaM.
Herein, we performed a detailed structural and biochemical comparative characterisation of the interaction of ACA8 with CML36 and CaM, respectively, aimed at identifying possible differences in the binding mode and in the target recognition mechanism between these two calcium sensors. The study was conducted by using two synthetic peptides corresponding to the known CaM-binding sites on ACA8 (ACA8BS1 and ACA8BS2)[2]. We demonstrated by native-PAGE analysis that CML36 and CaM recognise the same regions on ACA8 and bind with the same stoichiometry. Moreover, by nuclear magnetic resonance (NMR), fluorescence and circular dichroism (CD) spectroscopy, we investigated protein-target affinity and the conformational change associated with target binding. Notably, NMR analysis showed substantial differences in the structural rearrangements of the two proteins upon peptide binding, thus suggesting a different recognition mode.
Our findings will be crucial in understanding how CaM/CML proteins coexist and specifically contribute to plant cellular signaling.

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Session I · Protein Interactions and Dynamics

P13 - Oral Selected

TANC2 a possible dynamic regulator of CDKL5 protein levels

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Mutations in the protein kinase CDKL5 have been associated to diverse severe neurodevelopmental disorders (NDDs), including atypical Rett syndrome. CDKL5 is implicated in several neuronal processes, such as neuronal morphogenesis and excitatory synapse development. However, the molecular mechanisms whereby CDKL5 exerts its functions are still largely unknown. Knowledge of the molecular partners of CDKL5 may help identify targets for pharmacological intervention.
Our study demonstrated that the post-synaptic scaffold TANC2, recently emerging as a candidate gene for NDDs, directly interact with CDKL5 possibly regulating its PP1 mediated phosphorylation and its consequent proteasome degradation. Although TANC2 function in brain cells remains unclear, it seems to play a critical role in organizing different components of glutamate receptor complexes at PSD determining synaptic strength and plasticity [(1)].
The interaction among CDKL5-TANC2-PP1 proteins was assessed both by co-localization analysis in primary hippocampal cultures from E18 rats and neuroblastoma cell line, and immunoprecipitation from rat synaptosomes. By using yeast two-hybrid assays we determined that the CDKL5 - TANC2 interaction involve intrinsically disordered regions at their C-termini. Furthermore, TANC2 silencing was performed in SHSY5Y cells and we found that in absence of TANC2, CDKL5 protein level significantly increases. These findings support a critical role of TANC2 in CDKL5 protein availability and phosphorylation levels, which need to be tightly regulated for proper signaling of the synaptic plasticity, and suggest TANC2 as a possible target of therapeutic strategy.

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Session I · Protein Interactions and Dynamics

P14

How do proteins interact with ice? The case of EfcIBP

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Ice binding proteins (IBPs) are characterized by the ability to bind ice crystals and control their growth and shape. The interaction between IBPs and ice is translated into two main activities: the inhibition of ice recrystallization (IRI) and the increase of the gap of thermal hysteresis (TH). The functional part of IBPs is called ice binding site (IBS) and it is typically flat, relatively hydrophobic, and often characterized by the presence of threonine-rich repeats.
The bacterial IBP, *Efc*IBP, was identified by metagenomics analysis of the Antarctic ciliate *Euplotes focardii* and its associated bacterial consortium. *Efc*IBP has one of the highest IRI activity described to date[1] and its 3D structure, solved by X-ray crystallography, consists of a β-solenoid and an α-helix alongside the main axis of the protein. Structural analysis together with site-directed mutagenesis showed that EfcIBP binds ice crystals through two faces of the β-solenoid. This peculiarity not present in all IBPs makes *Efc*IBP a good model to study the interaction between IBPs and ice.

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Session I · Protein Interactions and Dynamics

P15

Intra vs. intermolecular communication in proteins revealed by Molecular Dynamics simulations: a GCAP1 story

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Guanylate Cyclase Activating Protein 1 (GCAP1) is a neuronal calcium (Ca^{2+}) sensor protein that regulates the phototransduction cascade in vertebrates upon subtle conformational changes, by switching between activator and inhibitor of the target guanylate cyclase (GC) in a Ca^{2+}-dependent manner. GCAP1 is also target of several mutations causing cone/rod dystrophies, degenerative retinal diseases ultimately leading to blindness. Here we carried out exhaustive molecular dynamics simulations of GCAP1 and determined the intramolecular communication pathways involved in the specific GC activator/inhibitor switch. The switch was found to depend on the Mg^{2+}/Ca^{2+} loading states of the three EF hands and on the way the information is transferred from each EF hand to specific residues at the GCAP1/GC interface. Post-translational myristoylation is fundamental to mediate long range allosteric interactions including the EF2-EF4 coupling and the communication between EF4 and the GC binding interface. The investigation of the functional role of key residues in the protein network topology revealed that some hubs are the target of retinal dystrophy mutations, suggesting that the lack of complete inhibition of GC observed in many cases is likely due to the perturbation of intra/intermolecular communication routes[1]. Moreover, since this protein was recently found to be a dimer[2] in solution and the dimerization interface partially overlaps the GC interface, we investigated the effects of the quaternary structure on the communication between EF hands and the target-regulating interface.

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Session I · Protein Interactions and Dynamics

S03

Predicting protein-protein interaction sites with computational approaches

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The identification of Protein-Protein Interaction (PPI) sites is a key step for understanding protein function in the context of cell complexity. Computational tools are useful in order to complement experimental methods in the attempt of accurately identifying PPI sites starting from protein structure or sequence.
In this context, we developed ISPRED4[1], an improved structure-based predictor of PPI sites on unbound monomer surfaces. ISPRED4 relies on machine-learning methods incorporating features extracted from protein sequence and structure. Cross-validation experiments were carried out on a new dataset that includes 151 high-resolution protein complexes, showing that ISPRED4 is one of the top-performing PPI site predictors developed so far. Moreover, ISPRED4 state-of-the-art performance was further assessed and confirmed on protein targets released during recent CAPRI experiments.
The prediction of PPI sites starting from protein sequence is an important but still challenging task. Few methods have been presented so far, achieving very limited prediction performance if compared with structure-based methods. We recently contributed in this field by developing ISPRED-SEQ, a novel approach based on advanced deep-learning techniques to predict PPI sites starting from protein sequence alone. The method has been benchmarked on two different datasets of proteins derived from literature. In these experiments, ISPRED-SEQ performance have been proven to be comparable to other state-of-the-art sequence-based methods.

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Session I · Protein Interactions and Dynamics

P16

Noble metals within ferritin nanocages: structural studies

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The encapsulation of Pt and Au-based anticancer agents within ferritin (Ft) is a promising way to enhance the selectivity of these molecules.[1] Well established metallodrugs like cisplatin[2], carboplatin[3] and poorly soluble Au-based drugs[4-6] have been trapped within the Ft nanocages. The adducts of these compounds with Ft are moderately selective towards cancer cells, when compared to non-malignant cells. Gold-encapsulated nanocarriers have a cytotoxic effect on different human cancer cells, induce oxidative stress activation and apoptosis. The metal-compound loaded proteins were characterized by UV-vis absorption spectroscopy, circular dichroism, inductively coupled plasma mass spectrometry and X-ray crystallography. Structural data show that the compounds often degrade upon encapsulation within the protein cage and that metal-containing fragments bind Cys or His residue side chains. Altogether our data indicate that encapsulation of metal-based drugs within Ft nanocages is a promising strategy to deliver these molecules to their final targets.

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Session I · Protein Interactions and Dynamics

P17

PTMs study of sea urchins' toposome protein induced by environmental factors through a proteomic approach

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Ocean acidification and exposure to generic pollutants are considered one of the most pervasive human impacts on marine life. In recent decades, the use of aquatic organisms as molecular biomarkers has acquired relevance. Biomarkers can act as prognostic tools for increased levels of pollution, since a variety of responses have been shown, ranging from physiology to gene and protein expression. In particular, the sea urchin *Paracentrotus lividus* is recognized as a model to study the response of marine organisms to environmental stress.
In *Paracentrotus lividus* the protein toposome, and its post-translational regulation plays important roles in the gametogenesis and embryo development[1]. Since protein nitration induced by natural or anthropogenic stressors, can affect the structure and the function of sea urchins' toposome, the protein was purified from gonads' animals collected in different conditions and analysed to detect possible nitration events.
Identification of protein and peptides with nitrated residue was carried out by a nanoLTQ-Orbitrap Velos mass spectrometer. Database searching was performed using the Sequest search engine of Proteome Discoverer 1.4.
This study highlights for the first time that post-translational modifications induced by environmental factors can affect the function of the toposome in sea urchins. Our data[2] suggests that NO-induced modifications of toposome can be considered as an environmentally-driven trait change, likely transmitted to future generations, with consequences at the population and ecosystem levels.

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Session I · Protein Interactions and Dynamics

P18

On the pLG72-D-amino acid oxidase interaction and schizophrenia susceptibility

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In the brain, the human flavoenzyme D-amino acid oxidase (hDAAO) degrades D-serine (D-Ser), an activator of N-methyl-D-aspartate receptors (NMDAR). pLG72, a small protein present in primates only, interacts with hDAAO, promoting its inactivation and degradation and thus modulating D-Ser cellular level[1]. The rs2391191 (M15) SNP in G72 results into the R30K substitution and has been correlated with the decreased thickness of the brain cortex in schizophrenic patients and with poorer episodic memory function.
The R30K substitution does not affect pLG72 conformation, its homodimeric state and the binding of hydrophobic ligands. Compared to the wild-type pLG72, the R30K variant shows a tighter hDAAO binding and a faster inactivation of the flavoenzyme thus yielding an increase in cellular (D/D+L)-serine in human glioblastoma U87 cells stably expressing pLG72. However, at the cellular level the R30K pLG72 is significantly more prone to degradation than the R30 variant (half-life of 13.6 vs. 23.9 min): a faster turnover of R30K pLG72 yields a lower cellular concentration of this hDAAO negative modulator and thus lead to a less effective inactivation of the flavoenzyme[2].
In turn, expression of R30K pLG72 generates a decrease in D-Ser cellular concentration, a condition that generates NMDAR hypofunction, which is a central component in the glutamatergic hypothesis of schizophrenia onset.

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Session I · Protein Interactions and Dynamics

P19

Extracellular Proteases from B. subtilis Trigger Blood Coagulation

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Mounting clinical evidences suggest a positive correlation between infectious diseases and higher thrombotic risk.[1] Blood coagulation comprises numerous zymogen/protease activation reactions that culminate in the conversion of Prothrombin (ProT) (Ala1-Glu579) into α-thrombin (αT) by factor Xa, which attacks ProT at Arg271 and Arg320. Depending on which bond is cleaved first, two different intermediates are generated: prethrombin-2 (Pre2) (first cleavage at Arg271) or meizothrombin (MZ) (first cleavage at Arg320).[2] We have recently found that subtilisin, a serine protease secreted from *Bacillus subtilis*, cleaves ProT to the novel active species σPre2, a Pre2 derivative nicked at the peptide bond Ala470-Asn471.[3] Although σPre2 is different from natural αT, yet it is able to coagulate blood by generating fibrin and agglutinating platelets. Notably, the entire extracellular protease activity of *B. subtilis* is ascribed to subtilisin (75%), and neutral protease (NP) (25%), a zinc-dependent hydrolase. Likewise, here we show that NP quantitatively attacks ProT at the peptide bond Asn162-Leu163 and at Asn471-Val472, generating a novel MZ-like active species, we named npPre1. The cleavage sites were identified by SDS-PAGE and mass spectrometry, and the functional properties of npPre1 characterized on synthetic (S2238) and physiological (fibrinogen) substrates.
In conclusion, our findings unravel that the whole proteolytic repertoire of *B. subtilis*, an abundant commensal in the intestinal microbiome, can directly trigger blood coagulation. These findings have important implications in inflammatory diseases of the enteric tract, whereby an increase of the permeability of the inflamed mucosa may lead to a leakage of the proteases secreted from *B. subtilis* in the bloodstream, where they can trigger abnormal blood coagulation.

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Session I · Protein Interactions and Dynamics

S04

Altered intracellular pathways in inherited disorders of vitamin B12 metabolism

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Vitamin B12 (B12; also known as cobalamin) is a B vitamin that has an important role in cellular metabolism, especially in DNA synthesis, methylation and mitochondrial metabolism. Deficiency is caused by either inadequate intake, inadequate bioavailability, malabsorption or inherited disease of intracellular Vitamin B12 metabolism. Inherited disorders associated with mutations in genes encoding proteins involved in intracellular B12 metabolism are designated complementation groups CblA to CblJ by complementation phenotyping of fibroblasts.
Methylmalonic Acidemia with Homocystinuria, cobalamin deficiency type C (cblC) (MMACHC) is the most common inborn error of cobalamin metabolism.
Despite a multidrug treatment, the long-term follow-up of early-onset patients is often unsatisfactory, with progression of neurological and ocular impairment.
We have studied the in-vivo proteome of control and MMACHC lymphocytes. A deregulation in proteins involved in cellular detoxification, especially in glutathione metabolism was found. In addition, relevant changes were observed in the expression levels of proteins involved in intracellular trafficking and protein folding, energy metabolism, cytoskeleton organization and assembly.
We then investigated MMACHC interactome using an Affinity-Purification Mass spectrometry experimental approach.
Our findings expand current understanding of the cblC disease and may ignite new research and therapeutic strategies to treat this disorder.

Session I · Protein Interactions and Dynamics

P20

Production and biochemical characterization of the Neuronal Calcium Binding Protein NECAB1

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Neuronal Calcium Binding Proteins (NECABs) are a novel family of human proteins composed by three members (NECAB1, NECAB2 and NECAB3) which share EF-hand domains at the N-terminus, a central highly conserved region called NECAB homology region (NHR) and an antibiotic biosynthesis monooxygenase domain at the C-terminus, which is a bacterial domain of unknown function in mammals[1]. To date, the role of this subclass of proteins is unclear, although some putative interactors have been recently identified[2].
Herein, we describe the production and the biochemical characterization of NECAB1 isoform in order to gain insights into the structural organization of this uncharacterized protein and to evaluate its capability to bind calcium. Towards these objectives, we have successfully expressed in *E.coli* recombinant NECAB1, obtaining milligram quantities of pure and stable protein, and explored its molecular architecture employing a combination of biochemical and biophysical techniques, including circular dichroism, fluorescence spectroscopy, and hydrodynamic analysis. NECAB1 was found to exist as a soluble high order oligomer, under native conditions, and to exhibit hallmark features of coiled-coil helices with a compact hydrophobic core. Moreover, thermal and urea-induced unfolding experiments indicated that NECAB1 follows the typical denaturation behavior of a protein with a well-defined and compact conformation. Importantly, no differences in the protein stability, conformation and oligomeric state were observed in the presence or in the absence of Ca^{2+}.
On the basis of these observations, along with a molecular modelling prediction, we propose that coiled-coil helical formation is an essential structural feature of NECAB1. Coiled-coil helices are frequently encountered motifs in protein–protein interactions involving subunit oligomerization, therefore they could have a crucial role in the interaction of NECAB1 with its target(s).

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Session I · Protein Interactions and Dynamics

P21

Preferential binding of Mg^{2+} over Ca^{2+} to CIB2 triggers an allosteric switch impaired in Usher syndrome type 1J

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Calcium- and integrin- binding protein 2 (CIB2) is a ubiquitous Ca^{2+} and Mg^{2+} sensor protein whose function is not completely clear. Recently, a CIB2 variant, namely p.E64D (p. Glu64Asp), was found to be associated to Usher syndrome J1 (USH1J), a disease leading to hearing loss and blindness. The present study reports a detailed biochemical *in vitro* characterization of recombinant wild type (WT) CIB2 and p.E64D variant using size exclusion chromatography and spectroscopic techniques, i.e., dynamic light scattering, circular dichroism, nuclear magnetic resonance and fluorescence. It was found that WT CIB2 does not possibly work as a Ca^{2+} sensor under physiological conditions, because its affinity for Ca^{2+} (K_d^{app} = 0.5 mM) is too low for detecting normal intracellular levels. Instead, WT CIB2 has a fairly high affinity for Mg^{2+} (K_d^{app} = 290 μM), and it is probably Mg^{2+} -bound under physiological conditions. CIB2 forms a non-covalent dimer under conditions that mimic the physiological ones. The Mg^{2+} binding to the WT protein creates a long range allosteric communication between the residue E64, located at the N-terminal domain, and the metal cation binding site EF3, located at the C-terminal domain. The conservative p.E64D mutation breaks up such inter-domain communication resulting in the impaired ability of CIB2 to switch to its Mg^{2+}-bound form. The ability to bind the target integrin α7b peptide was substantially conserved for p.E64D, therefore the present study suggests that the molecular defect associated to USH1J resides in its inability to sense Mg^{2+} and adopt the required conformation.

Session II · Amyloids and Intrinsically-Disordered Proteins

P22

Using gold nanoparticles to assess protein accessibility and protein-protein interactions in complex networks

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Geometric and structural features of protein networks are difficult to assess when networks lack a defined structure and when solubility of the involved proteins is limited. Solubility issues prevent using spectroscopic approaches, and amorphous structures make classical/advanced microscopic approaches almost inefficacious.
We took advantage of the high thiol reactivity of the surface of Au nanoparticles (AuNPs) to test accessibility of protein thiols in a complex model network, involving water-insoluble gliadins and glutenins in durum wheat semolina, where proteins interact through both hydrophobic interactions and disulfide bonds. After removal of non covalently bound proteins, proteins bound to the AuNPs (either directly or through "piggybacking" on other proteins) were identified by MS/MS.
No gluten proteins were bound to AuNPs (20 nm average size) in the absence of pre-treatments (low molarity urea or detergents) that loosened hydrophobic interactions in the grain proteins network. Gluten proteins bound in network-loosening conditions included both glutenins (containing both free cysteines and intramolecular disulfides), but also gliadins (having no free cysteines and many intramolecular disulfides), proving that the two protein classes are interacting in the grain through intermolecular disulfides.
These preliminary results pave the way to using AuNPs of different size for testing the nature of interacting proteins, the chemistry of their interaction, and the geometrical features of the resulting network whenever cysteine-containing proteins are involved. This is obviously relevant to many food-related systems, but may be useful also for addressing the chemical and geometrical features of several pathologically relevant protein aggregates.

Session II · Amyloids and Intrinsically-Disordered Proteins

P23 - Oral Selected

How to design an (in)soluble protein tag: some insights from synthetic intrinsically disordered proteins

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An intrinsically disordered protein (IDP) can be defined as a conformationally fluctuating ensemble endowed with a few structural constraints with respect to globular "ordered" proteins. Besides this, IDPs share high solubility due to their amino acid composition, which is depleted of hydrophobic residues and rich of proline and charged residues. Our work was focused on describing how charged residues and environment pH influences IDP solubility.
We carried out a systematic exploration of pH effects on a set of synthetic IDPs just differing in their net charge per residue (NCPR). Although keeping their character of intrinsic disorder, our synthetic proteins exhibit different solubility/aggregation propensity, highly responding to environment pH fluctuations.
Overall, NCPR, more than isoelectric point or net charge, is strongly affecting IDP solubility 1, irrespective of charge relative position and representing a useful parameter to design ad hoc sequences whose (in)solubility can be easily predicted.

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Session II · Amyloids and Intrinsically-Disordered Proteins

P24

Autophagy activation induced by the first genetic variant of β2 microglobulin oligomers

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It is widely recognized that the mechanism of amyloid aggregate cytotoxicity requires the primary interaction between the aggregates and the cell membrane, resulting in functional and/or structural perturbation of the latter.
Moreover, the presence of toxic aggregates inside or outside the cells, together with their interaction with cell membranes can impair a number of functions ultimately leading to cell death by apoptosis or autophagy.
Autophagy is the key process involved in the homeostasis of proteins, lipids and organelles that help to clean the cell from an exogenous or endogenous material that is useless or harmful to cells. Generally, autophagy is induced by cell starvation, but other stresses can be caused by the activation of this process, such as the presence of poorly folded protein deposits or the disposal of old cellular organelles.
My study is focused on understanding the signaling pathway responsible for the autophagic process induced by the interaction of the first genetic variant of β2-microglobulin (B2M-D76N) aggregates with cell membranes.
I have investigated the interaction process of amyloid aggregates at different stages of aggregation with specific membrane sites characterized by the presence of GM1 and the consequent activation of the signaling cascade responsible for the autophagic process.
In fact, data acquired recently by Western Blot and confocal microscopy techniques have confirmed the activation of autophagy in SH-SY5Y cells treated with B2M-D76N oligomers. The formation of autophagosomes and the increase of LC3-II, were observed at five hours of treatment. Moreover, a precise autophagic pathway has been identified that involves AKT and GSK3β upstream of the mTOR complex, whose inhibition promotes the activation of autophagy, and P-S6 downstream. Finally, the latest experiments seem to indicate the involvement of the IGF1R receptor in the activation of the above described signaling pathway but this data needs further analysis.

Session II · Amyloids and Intrinsically-Disordered Proteins

S05

The amyloidogenic and Aβ scavenger states of transthyretin studied with high resolution intrinsic fluorescence and FRET

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Transthyretin (TTR) is an extracellular protein able to deposit into well-defined amyloid aggregates in pathological conditions known as senile systemic amyloidosis (SSA), familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC) and leptomeningeal amyloidosis (LMA). It also acts in the brain as a scavenger of the amyloid β (Aβ) peptide associated with Alzheimer's disease. In order to investigate the amyloidogenic partially folded state adopted by the protein under conditions promoting amyloid formation and the conformational state adopted by the protein while acting as an Aβ scavenger, we have used fluorescence resonance energy transfer (FRET) experiments coupled to highly resolved fluorescence spectroscopy in a monomeric variant of TTR (M-TTR), taking advantage of the presence of the natural highly fluorescent tryptophan residue at positions 41 (acting as a donor) and of a coumarin moiety covalently attached to Cys10 (acting as an acceptor). Trp41 is located in an ideal position as it is one of the residues of β-strand C, whose degree of unfolding is debated in the amyloidogenic state of the protein. We found that the amyloidogenic state has the same FRET efficiency as the fully folded state, indicating an unmodified spatial distance between Cys10 and Trp41, whereas the Aβ-bound state of M-TTR has a lower FRET efficiency, indicating a conformational change upon Aβ binding. Using highly resolved fluorescence spectroscopy we will also show that Trp41 adopts a number of conformational states (rotamers) in the folded state whose distributions are maintained in the amyloidogenic state. Overall, these FRET results indicate that it is possible, using a sort of "spectroscopic ruler", to monitor the change of the distance of a region of M-TTR as critical as that containing the observed Trp41 from other regions of the protein structure, following a given conformational change of interest, whereas the enhanced resolution of the intrinsic fluorescence spectra of the protein allows to detect the inherent conformational flexibility of such a region and to monitor its variation following the same conformational change.

Session II · Amyloids and Intrinsically-Disordered Proteins

S06

An NMR approach to structure and dynamics of human transthyretin

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Transthyretin (TTR) structure has been widely investigated in the last 40 years with an overrepresentation in the Protein Data Bank (PDB) that counts around 350 entries. In spite of our detailed knowledge of TTR tertiary and quaternary interactions, also in association with various ligands, our understanding of the dissociation mechanism leading to fibril formation is not fully understood at an atomistic level. It is modelled by exposure of the protein to non-physiological low pH *in vitro* (Colon et al. Biochemistry 1992) and is inhibited by small molecule compounds, such as the drug tafamidis (Bulawa et al. PNAS 2012). Recently, a new mechano enzymatic pathway of TTR fibrillogenesis *in vitro*, catalysed by selective proteolytic cleavage, which produces a high yield of genuine amyloid fibrils has been identified (Mangione et al. PNAS 2014; Marcoux et al. EMBO MM 2015). This pathway is efficiently inhibited only by ligands that occupy both binding sites in TTR (Kolstoe et al. PNAS 2010; Verona et al. Sci Rep. 2017).
The average of the coordinates root mean square deviation (RMSD) of the deposited X-ray structures also in the presence of ligands is lower than 1Å, indicating their substantial identity. The attempt of our work is to study the behaviour of TTR in solution by NMR spectroscopy and to correlate the obtained information with fibrillogenesis data.

Session II · Amyloids and Intrinsically-Disordered Proteins

P65

Structural determinants in ApoAI amyloidogenic variants explain improved cholesterol metabolism despite low HDL levels

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Amyloidoses are disease characterized by aggregation of misfolded proteins in fibrils. Specific gene mutation in Apoliprotein AI (ApoAI) leads to variants responsible for a systemic hereditary amyloidosis in which protein fibrils accumulate in different organs, causing their failure. Previous studies have shown that ApoAI mutations affect proteins stability, proteolytic susceptibility and aggregation propensity. Several ApoAI variants are also associated with hypoalphalipoproteinemia, low ApoAI and HDL-cholesterol plasma levels; but patients affected by ApoAI-related amyloidosis don't show a higher risk of cardiovascular diseases (CVD). Inspecting structural features, lipid binding properties and functionality of four ApoAI amyloidogenic variants, this work means to clarify the paradox observed in patients. A complementary proteolysis approach coupled to mass spectrometry was employed to map the exposed and flexible regions in ApoAI proteins, thus allowing the detection of conformational alterations in the different lipid free and bound variants. Results showed that the position of the mutation in the ApoAI amyloidogenic variants affects the molecular structure of HDL particles. Moreover, lipidation increases ApoAI proteins stability but all the amyloidogenic variants analyzed showed a lower affinity for lipids and different proteolytic pattern compared to lipid free mutants. Interestingly, the lower efficiency at forming HDL particles is compensated by a higher efficiency at catalysing cholesterol efflux from macrophages. The decreased affinity of ApoAI amyloidogenic variants for lipids, together with the increased efficiency in the cholesterol efflux process, could explain why, despite the low lipid levels, patients affected by ApoAI related amyloidosis do not show a higher CVD risk.

Session II · Amyloids and Intrinsically-Disordered Proteins

S07

Kinetics of protein aggregation

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This talk describes our efforts to elucidate the molecular mechanisms of protein aggregation as well as the dynamics of such systems and how these features connect to the biological roles that these structures can have in both health and disease. A particular focus will be on the development and use of tools and concepts from physical chemistry, in particular chemical reaction kinetics, to discover molecular assembly pathways in amyloid formation. Moreover we will discuss the development of new microfluidics approaches to study heterogeneous protein self-assembly and their application to explore the molecular determinants of amyloid formation from peptides and proteins.

Session II · Amyloids and Intrinsically-Disordered Proteins

P25

Structure based screening and validation of novel drugs against gelsolin amyloidosis: use of C. elegans to hunt proteotoxicity

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Agel amyloidosis is an orphan disease due to pathological aggregation of the protein gelsolin. First described in 1969, Agel has been long associated to only D187N/Y mutations. Since 2013, three novel pathogenic variants have been identified (N184K, G167R and P432R) and a sporadic form of the disease.
We recently obtained high resolution crystal structures for N184K[1], G167R[2] and D187N proteins. Structural and biophysical investigation revealed that mutations affect protein stability and flexibility, ultimately leading to protein degradation and deposition. Using these structures as target, we started an *in silico* screening for ligands able to stabilize the protein and prevent its aggregation.
In this context, we developed an assay to evaluate gelsolin proteotoxicity *in vivo* and the efficacy of the selected drug candidates. Employing the nematode *C. elegans* as "biosensor" capable of recognising biologically relevant assemblies of amyloidogenic proteins3, we investigated the toxicity of the different gelsolin isoforms. Rhythmic contraction and relaxation of the *C. elegans* pharynx, the organ deputized to feeding, are inhibited when worms are exposed to toxic solutes, as a survival mechanism that limits further intake of molecules recognized as harmful.
We observed that wild-type gelsolin induced a transient reduction of the *C. elegans* pharyngeal functionality. The presence of D187N, N184K or G167R mutation worse the protein toxicity causing a specific, dose-dependent and permanent impairment of pharyngeal contraction. A gelsolin nanobody, known to stabilize mutated gelsolin, when co-administered to C. elegans, counteracted the protein toxicity.
In conclusion, these data indicate that this nematode-based assay is a promising surrogate model for investigating the proteotoxicity of amyloidogenic gelsolin and for a rapid screening of structure-based designed therapeutic strategies.

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Session II · Amyloids and Intrinsically-Disordered Proteins

P26

Molecular insights into Oleuropein aglycone interference in α-synuclein aggregation process

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α-synuclein plays a key role in the pathogenesis of Parkinson's disease (PD); its deposits are found as amyloid fibrils in Lewy bodies and Lewy neurites, the histopathological hallmarks of PD. Amyloid fibrillation is a progressive polymerization path starting from peptide/protein misfolding and proceeding through the transient growth of oligomeric intermediates widely considered as the most toxic species. Consequently, a promising approach of intervention against PD might be preventing α-synuclein build-up, misfolding and aggregation[1]. A possible strategy involves the use of small molecules able to slow down the aggregation process or to alter oligomer conformation favoring the growth of non-pathogenic species.
Here, we show that oleuropein aglycone (OleA)[2], the main olive oil polyphenol, exhibits anti-amyloidogenic power *in vitro* by interacting with, and stabilizing, α-synuclein monomers thus hindering the growth of on-pathway oligomers and favoring the growth of stable and harmless aggregates with no tendency to evolve into other cytotoxic amyloids. We investigated the molecular basis of such interference by both biophysical techniques and limited proteolysis; aggregate morphology was monitored by electron microscopy. We also found that OleA reduces the cytotoxicity of α-synuclein aggregates by hindering their binding to cell membrane components and preventing the resulting oxidative damage to cells.

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Session II · Amyloids and Intrinsically-Disordered Proteins

P27

A step towards the comprehension of the insurgence of the Congenital Central Hypoventilation Syndrome

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Polyalanine expansions in the 20-residues region of the C-terminus of the human PHOX2B gene, a key regulator of autonomic nervous system development, are the major mutations responsible for the Congenital Central Hypoventilation Syndrome (CCHS, OMIM ID: 209880)1. This neurodevelopmental disorder is characterized by a failure in the autonomic control of breathing. Elongation of the alanine stretch in PHOX2B determines a protein with altered DNA-binding, transcriptional activity and nuclear localization, and the possible formation of cytoplasmic aggregates[1,2].
Here we report on the heterologous expression and the purification of the wild-type PHOX2B protein and the pathological form presenting in the alanine stretch an expansion of seven alanines as well as a comparative study of their biochemical properties. By a multidisciplinary approach the different physical-chemical features of the two recombinant proteins were analyzed. In addition, AFM studies were performed on the two proteins highlighting a propensity for the pathological variant to develop amyloid-like aggregates.
These results provide novel insights not only into the effects of the alanine tract expansion on PHOX2B folding but also pave the bases for the comprehension of the insurgence of the CCHS.

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Session II · Amyloids and Intrinsically-Disordered Proteins

P28

Subtilisin from Bacillus subtilis cleaves Human Transthyretin (hTTR) and generates the amyloidogenic fragment hTTR(59-127)

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hTTR is an abundant homo-tetrameric plasma protein involved in the transport of thyroxine and retinol-binding protein. hTTR is also a major component of amyloid protein deposits found in different disorders associated to the formation of amyloid fibrils. Bellotti and co-workers have recently reported that proteolysis of wild-type and mutant hTTR by bovine pancreas trypsin can trigger amyloid formation.[1,2] However, it is unlikely that trypsin and hTTR might come into contact *in vivo*.
Here, we show for the first time that, under naïve conditions (pH 7.4, 37°C), subtilisin cleaves hTTR at Leu58-Thr59 bond, generating fragment hTTR(59-127), which is resistant to further cleavage and forms amyloid-like fibrils, as documented by turbidimetric analysis and thiophlavin-T binding assay. hTTR(59-127) was purified to homogeneity by RP-HPLC and chemically characterized by mass spectrometry (7757.2±0.2 kDa). Purified hTTR(59-127) was dissolved in Tris-buffered saline, pH 7.4, and after only 15-min incubation protein aggregates were visible. Transmission Electron Microscopy analysis of hTTR(59-127) deposits revealed the presence of ordered amyloid-like fibrils (length: 200±50 nm; thickness: 20±5 nm).
These findings might have relevant implications in hTTR-based amyloidogenic complications, often associated to Inflammatory Bowel Diseases (IBD).[3] Subtilisin is a serine protease (not belonging to the trypsin family) secreted from *B. subtilis* which is an abundant commensal in the gut microbiota. In IBD, the permeability of the intestinal mucosa is much increased,[4] possibly allowing translocation of subtilisin to the bloodstream where the protease might cleave hTTR to hTTR(59-127) and trigger fibril formation.

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Session II · Amyloids and Intrinsically-Disordered Proteins

P29 - Oral Selected

Conformational dynamics in crystals reveal the molecular bases for D76N Beta-2 microglobulin aggregation propensity

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Spontaneous aggregation of folded and soluble native proteins in vivo is still a poorly understood process. A prototypic example is the D76N mutant of beta-2 microglobulin (2m) that displays an aggressive aggregation propensity. Here we investigate the dynamics of 2m by X-ray crystallography, solid-state NMR, and molecular dynamics simulations to unveil the effects of the D76N mutation. Taken together, our data highlight the presence of minor disordered sub-states in crystalline 2m. The destabilization of the outer strands of D76N 2m accounts for the increased aggregation propensity. Furthermore, the computational modeling reveals a network of interactions with residue D76 as a keystone: this model allows predicting the stability of several point mutants. Overall, our study shows how the study of intrinsic dynamics in crystallo can provide crucial answers on protein stability and aggregation propensity. The comprehensive approach here presented may well be suited for the study of other folded amyloidogenic proteins.

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Session II · Amyloids and Intrinsically-Disordered Proteins

P30

Concurrent structural and biophysical traits link with immunoglobulin light chains amyloid propensity

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Light chain amyloidosis (AL), the most common systemic amyloidosis, is caused by the overproduction and the aggregation of monoclonal immunoglobulin light chains (LC) in target organs. Due to genetic rearrangement and somatic hypermutation, virtually, each AL patient presents a different amyloidogenic LCs [1]. Because of such complexity, the fine molecular determinants of LC aggregation propensity and proteotoxicity are still unclear; significantly, their decoding requires investigating large sets of cases. Aiming to achieve generalizable observations, we systematically characterized a pool of thirteen sequence-diverse full length LCs. Eight amyloidogenic LCs were selected as responsible for severe cardiac symptoms in patients; five non-amyloidogenic LCs were isolated from patients affected by multiple myeloma. Ours is a comprehensive biophysical and structural approach, consisting of spectroscopic techniques, limited proteolysis, and X-ray crystallography. Our data show that low fold stability and high protein dynamics correlate with amyloidogenic LCs, while hydrophobicity, structural rearrangements and nature of the LC dimeric association interface (as observed in seven crystal structures here presented) do not appear to play a significant role in defining amyloid propensity [2]. Based on the structural and biophysical data, our results highlight concurrent fundamental biophysical properties drive LC amyloid propensity.

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Session II · Amyloids and Intrinsically-Disordered Proteins

P31

Neuroserpin Misfolding and FENIB: Mechanism and Inhibition Processes

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Neuroserpin (NS) is a serin protease inhibitor specifically expressed in the brain that undergoes polymerisation upon point mutation. NS polymerisation is directly correlated with the familial encephalopathy with neuroserpin inclusion bodies (FENIB) onset. In literature there is increasing evidence of a crucial role for glycosilation in preventing polymerisation. All the biochemical and biophysical characterizations performed since now are conducted on NS purified by *Escherichia coli* cells, so lacking this postranslational modification. Here, we present a novel system to overcome this lacking of information: expression of NS in *Leishmania tarentolae* cells. So far, a pharmacological treatment of FENIB remains an unmet challenge even if we recently reported a positive effect of embelin (EMB) on NS conformers and polymerisation. EMB destabilizes all known NS conformers, specifically binding to NS molecules without unfolding the NS fold or inhibiting its proteolytic activity. In particular, NS polymers disaggregate in the presence of EMB, and their formation is prevented. It acts firstly by inhibiting the NS polymerisation associated to FENIB, and secondly by potentially antagonizing metastatic processes facilitated by NS activity in the brain. Unfortunately its action is associated to a poor solubility in acqueous buffer and exerts a not negligible cellular toxicity so the need for active but soluble and safer EMB-like compounds.

Session III · Biocatalysis: mechanisms and applications

P32

Light dependent redox catalysis by Photosystem I complexes encapsulated in organic nanoparticles

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Photosystem I (PSI) is a pigment binding multi-subunit protein complexes involved in photosynthesis. PSI is localized in the thylakoid membranes and catalyze the electron transfer reaction from plastocyanin to ferredoxin, as one of the main steps involved in conversion of light energy into chemical energy. PSI is highly efficiency with a photochemical efficiency close to one. Several attempts have doing in the past in order to exploit the high efficiency and high stability of PSI in an extra-cellular context in order to catalyze electron transfer reactions: in this work we present an innovative solution for exploiting the photochemical properties of PSI, by encapsulation of PSI complexes in organic nanoparticles. Nanoparticles offer a protected environment to the encapsulated molecule, giving it the possibility of preserving its functional properties and studying how they change over time. In this work the complete characterization, both morphological and functional, of nanostructures obtained by encapsulation of PSI complexes purified from higher plants with PLGA (poly lactic-co-glycolic acid) polymer is presented. The results obtained by transient absorption and time-resolved fluorescence demonstrate that encapsulated PSI were characterized by an higher photochemcial activity compared to PSI complexes in detergent solution. Moreover, encapsulated PSI maintained the high efficiency observed for several weeks even if exposed to very strong light, being more stable compared to PSI in detergent solution. Finally, the nanostructures obtained by encapsulated PSI were able to catalyze light dependent redox reactions with electron acceptors and donors outside the nanostructures Potential application of these PLGA encapsulated PSI in different fields are thus presented and discussed.

Session III · Biocatalysis: mechanisms and applications

S08

Intra-site differential inhibition of enzymes: the case of aldose reductase

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Aldose reductase (ALR2; EC 1.1.1.21) is an NADPH dependent oxidoreductase which catalyzes the reduction of a wide variety of hydrophilic as well as hydrophobic aldehydes. ALR2 is the first enzyme of the so-called polyol pathway that allows the conversion of glucose into sorbitol, a polyalcohol that is then oxidized to fructose by sorbitol dehydrogenase. Under normoglycemic conditions, only a little quote of glucose is converted to sorbitol, due to the low affinity of ALR2 for glucose; instead in pathological conditions of hyperglycemia, glucose can enter polyol pathway. Numerous studies have shown that the reduction of glucose to sorbitol leads to a series of undesirable events: the osmotic imbalance due to sorbitol accumulation, the decrease of antioxidant defenses due to NADPH oxidation and the induction of conditions favoring protein glycation are considered responsible for a series of long term complications of diabetes such as retinopathy, cataract, nephropathy and neuropathy. The role of ALR2 in the onset of diabetic complications has made this enzyme the possible target for the development of molecules able to inhibit its activity. Despite the considerable effort devoted in the last decades for the synthesis of several potent inhibitors of ALR2, virtually all synthesized compounds failed as drugs for the treatment of diabetic complications. This failure may be due to several reasons from the low bioavailability of the inhibitor to the severe side effects, but it may also be related to the ability of ALR2 to reduce toxic aldehydes, products of oxidative stress.

In recent years we proposed an alternative approach to the inhibition of ALR2 suggesting the possibility of an intra-site differential inhibition of the enzyme through molecules able to preferentially inhibit the reduction of either hydrophilic or hydrophobic substrates. The rationale and examples of this new generation of aldose reductase “differential” inhibitors (ARDIs) are presented.

Session III · Biocatalysis: mechanisms and applications

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A versatile H2O2 dependent biocatalyst for conversion of aromatic compounds and production of drug metabolites

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Cytochromes P450 are heme-thiolate enzymes representing a large superfamily of proteins widely diffused in organisms from all kingdoms of life including viruses.
In the canonical P450 catalytic cycle, two electrons are donated at discrete points in the cycle via a redox partner protein (using NAD(P)H), then two successive protonations occur in order to activate the iron to a highly reactive form which oxidize the substrate.
However, some P450s are known to catalyse substrate oxidation using the "peroxide shunt", in which hydrogen peroxide activates directly the iron with several advantages.
We show here a versatile H2O2 dependent biocatalyst redesigned from the heme domain of CYP116B5, a self-sufficient class VII P450 which was proven to be a valid alternative to the conventional route of P450 catalysis. This enzyme was functionally expressed in *Escherichia coli* and purified with high yield. A full characterization of the enzyme using UV-Vis spectroscopy, spectroelectrochemistry and DSC analysis was performed.
The results demonstrated interesting peculiarities in comparison with other well studied P450s such as an unusual high redox potential and the high resistance to H2O2 therefore makes this enzyme a valuable candidate for performing catalysis through the peroxide shunt obviating the need for the expensive NAD(P)H cofactor.
The reactions performed by this enzyme include hydroxylation of aromatic rings and N-dealkylation of tamoxifen which are all interesting targets for both bioremediation and human drug metabolites' production.
In addition, this system was further optimized for waste water pollutant removal in combination with other oxygenases. Excellent results were obtained with two phenolic pollutants where o- cresol and 4-Chloro-3-methylphenol where degraded in water with removal percentage of 86% and 80% respectively.
This system paves the way for the use of CYP116B5 for large scale human metabolite production and in the bioremediation field.

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Lytic Polysaccharide Monooxygenases: new players in biomass breakdown

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Plant polysaccharides are the most abundant biopolymers on Earth; ones with major involvement in human health and diverse biocatalytic processes, notably biofuels. The search for new enzymes to improve the efficiency of second-generation bioethanol production is a major academic and industrial goal. Recent years have seen the discovery and application of (lytic) polysaccharide monooxygenases (L)PMOs – shown in 2011 to be Cu-dependent monooxygenases utilizing a signature 'histidine-brace' motif to coordinate the mononuclear Cu ion.[1] LPMOs act, primarily, on recalcitrant polysaccharides[2] such as chitin, cellulose and xylan,[3] with one LPMO family starch-specific.[4]
In this lecture I shall review the, often-perplexing, history and discovery of LPMOs from their early origins as "non-catalytic" proteins, the seminal discovery of their oxidative mode-of-action,[5] through confusion over metal-ion identity to more recent work on their substrate complexes.[6] Recent times have witnessed the discoveries of new families of LPMO, from diverse linages including ancient insects[7] and this ever-expanding world of LPMOs provides a major toolkit for the degradation of polysaccharides otherwise highly refractory to enzymatic attack.

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Properties of RNase A monomer and dimers depend on the oligomerization pathway followed

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Bovine pancreatic ribonuclease A (RNase A, 13.7 kDa), the "pancreatic-type" ribonuclease super-family proto-type, is able to oligomerize through a 3D domain swapping (3D-DS) mechanism involving its N- and/or C-termini[1]. RNase A oligomerization takes place when the native monomer is lyophilized from 40% acetic acid solutions[2] or when it is thermally incubated at high concentration in various solvents, such as 20 or 40% EtOH[3].
The 3D-DS mechanism is shared by several amyloidogenic proteins: indeed, human prion protein, cystatin C and β2-microglobulin form domain-swapped dimeric and oligomeric precursors of amyloid fibrils. Furthermore, the toxicity of the oligomers of proteins involved in proteopathies can depend on the pathway through which oligomers form[4].
Hence, although RNase A is not amyloidogenic, we investigated here if the two incubation methods used to induce RNase A self-association may produce different structural and/or functional differences in the oligomers formed. The RNase A monomers and N- and C-swapped dimers recovered after 40% acetic acid lyophilization or 60 °C thermal incubation in 20 or 40% EtOH, were purified through cation exchange chromatography. Their structural differences were studied with nondenaturing cathodic PAGE, far- and near-UV CD, fluorescence measurements upon ANS binding, and limited proteolysis with subtilisin. In addition, we studied with UV-CD and MS the differences emerging between the untreated monomer and the ones recovered after applying the mentioned protocols. Finally, also the enzymatic activity of the different RNase A monomers and dimers have been investigated, detecting interesting differences, while the measure of their cytotoxic activity is still ongoing.

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Structural investigations and substrate specificity of *T. litoralis* trans-3- Hydroxy-L-proline dehydratase

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L-Hydroxyproline is a key component of collagen, and the isomer trans-3-Hydroxy-L-proline (T3LHyp) constitutes about 10% of mammalian collagen. The conversion of T3LHyp into proline occurs through a metabolic pathway that is evolutionarily conserved across the three domains of life and consists in a two-steps reaction involving a T3LHyp dehydratase enzyme (EC 4.2.1.77) followed by a reductase enzyme. The T3LHyp dehydratase enzyme removes the hydroxyl group from the T3LHyp substrate without the involvement of any cofactor [1].
We have solved the crystal structure of the archaea *T.litoralis* T3LHyp dehydratase in the uncomplexed form and in complex with its natural substrate T3LHyp. The overall structure shows an "open" and a "close" conformation depending on the absence or presence of the substrate, respectively. Moreover, the structure reveals previously undetermined roles of key residues, conserved among the dehydratase family, involved in the specific recognition of T3LHyp.
We present here the first structure of a member of the hydroxyproline dehydratase protein family that could be used as a valid model for determining the substrate specificity of the human ortholog, a key enzyme for the metabolic degradation of T3LHyp in humans [2].

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Sulfur-containing amino acid metabolism of the human pathogen Toxoplasma gondii: cystathionine β-synthase

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The protozoan parasite T. gondii possesses the genes coding for a cystathionine β-synthase (TgCBS) and a cystathionine γ-lyase, as well as for a cysteine synthase, key enzymes of the reverse transsulfuration and assimilatory pathways, respectively. These three enzymes were recently identified as oocyst/sporozoite-specific proteins, opening new possibilities for the identification of novel good target candidates for anti-parasite drugs[1].
CBS catalyzes the pyridoxal 5′-phosphate (PLP)-dependent condensation of L-homocysteine with L-serine to form L-cystathionine. CBS in higher organisms (human and *D. melanogaster*) contains an N-terminal extension that forms a heme-binding domain, which is not observed in TgCBS. Similarly to human enzyme, TgCBS was found to contain the C-terminal CBS regulatory domain, which is a region characterized by two hydrophobic motifs designated "CBS domains"[2, 3].
Herein, we describe the overexpression in *E. coli* and the kinetic characterization of the putative CBS from *T. gondii*. We found that the enzyme shows CBS activity with L-serine and, to a letter extent, with O-acetyl-L-serine. TgCBS is also involved in H_2S generation, showing a marked preference for H_2S production via β-replacement of L-cysteine by L-homocysteine. The alternative H_2S-generating reaction, i.e. the condensation of two molecules of L-cysteine to generate L-lanthionine, is quantitatively less significant. Notably, TgCBS does not show response to S-adenosyl-L-methionine. Moreover, we have studied the role of CBS domains of TgCBS by generating a truncation mutant spanning residues 359-520. We expressed the truncation mutant in *E. coli* and determined the effect of deletion on activity, oligomeric status, and stability. Characterization of this deletion mutant sheds new light upon the function and organization of the catalytic and regulatory regions of TgCBS.

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Exploitation of monoxygenases in biocatalysis

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Two different classes of monoxygenases, respectively containing heme and flavin isolated and cloned in our lab, will be illustrated in their properties and biotechnological applications, ranging from their *in vitro* hydrogen-peroxide driven-catalysis, to electrochemical or in vivo whole cells synthesis of drug intermediates and metabolites.
Firstly, the heme domain of CYP116B5 from *A. radioresistens*, a self-sufficient class VII P450 with a high reduction potential and a high resistance to hydrogen peroxide, is a good candidate for peroxide-drive catalysis. Although its role in *A. radioresistens* is still unknown, its activity on different substrates sharing an aromatic scaffold, makes it an interesting candidate for applications in bioremediation and human drug metabolite production.
As a second example, the human flavin-containing monooxygenase 3 (hFMO3), an important phase-1 liver drug metabolizing enzyme, is shown. It catalyzes the oxygenation of a variety of soft nucleophilic molecules possessing highly polarizable electron lone pairs on their heteroatoms, such as those present in benzydamine, sulindac sulfide, tamoxifen, and aurora kinase inhibitors. Data on catalysis by direct electrochemistry on glassy carbon as well as gold electrode are shown, highlighting the different activities present in different human groups with important personalized medicine implications. In particular, a hFMO3-bioelectrode platform was obtained in a nanoscaled system, based on graphene oxide on glassy carbon electrodes. This same enzyme was also expressed in a whole cell format, for the production of the N-oxide metabolites of tamoxifen, benzydamine, clomiphene, dasatinib, GSK5182 and tozasertib.
Finally, *A. radioresistens* Baeyer-Villiger monooxygenase (Ar-BVMO) is shown not only to be capable of oxidizing two anticancer drugs metabolized by human FMO3, danusertib and tozasertib, but also to oxidize other synthetic drugs, such as imipenem, a clinically important antibiotic used in the treatment of MDR bacterial infections.

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Insights On S-Nitrosylation In Human Serine Racemase

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Human serine racemase (hSR) is a pyridoxal 5' phosphate dependent enzyme responsible for the synthesis of D serine, a neuromodulator of NMDA receptors. Its activity is modulated by several ligands, including ATP, divalent cations and protein interactors, which were shown to alter the tertiary and quaternary conformations of the protein.
hSR is 90% inhibited by S-nitrosylation at Cys113, a residue adjacent to the ATP binding site. We found that the time course of inhibition by S-nitrosylation is markedly biphasic, with a fast phase associated with the reaction of Cys113. Unlike the murine enzyme[1], two additional cysteine residues, Cys269, unique to the human orthologue, and Cys128 were also identified as S-nitrosylation sites through mass spectrometry and site-directed mutagenesis[2].
In the presence of both the allosteric modulator ATP and either malonate or glycine, which bind at the active site stabilizing a closed conformation — as observed by X-ray crystallography — S-nitrosylation at Cys113 is almost abolished in comparison to the protein in the presence of only ATP. This 100-fold slowdown of S-nitrosylation might be associated to an H-bond observed in the closed conformation – but not in the open one – between Asp318 and Cys113. Indeed, the D318N mutant undergoes fast S-nitrosylation even in the presence of ATP and glycine, indicating that the H-bond is likely the structural feature that dictates the conformation-dependent accessibility of Cys113 to S-nitrosylation.

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The rational-based directed evolution of L-amino acid deaminase, an enantioselective biocatalyst

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The L-amino acid deaminase from *Proteus myxofaciens* (PmaLAAD) is a membrane flavoprotein which catalyzes the deamination of L-amino acids (L-AA)[1]. This enzyme possesses several interesting properties for biotechnological applications: does not generate H_2O_2, is strictly enantioselective and can be produced as a recombinant protein. For these reasons, PmaLAAD represents a suitable biocatalyst to produce optically pure D-AAs (of high economic relevance since they are part of antibiotics, insecticides, etc.) by deracemization reactions or α-ketoacids from the corresponding L-AAs.
We recently demonstrated that PmaLAAD can be used as a biocatalyst in bioconversion of several natural or synthetic L-AAs, such as L-Phe, L-Met, L-DOPA or L-1-naphthylalanine (L-1-Nal)[2]. Previous attempts to evolve PmaLAAD by a fully-irrational protein engineering approach allowed only marginal improvement of its kinetic properties[3].
Here we employed a multidisciplinary approach (docking simulations, evolutionary analysis and site-saturation mutagenesis) to produce smaller, yet smarter, libraries of variants possessing altered substrate scope and improved kinetic parameters on substrates of biotechnological relevance. This approach allowed the identification of variants more active on large substrates such as L-1-Nal. The best variant (F318A/V412A/V438P-PmaLAAD), showed a specificity constant (the ratio between the specific activity on L-1-Nal, the desired substrate, vs L-Phe, the native one) 86.8-folds higher than the wild-type enzyme. Docking analysis of the putative complexes between the substrates and the variants showed the rationale for its increased activity on larger substrates. These variants perform better than the wild-type when used as biocatalysts in bioconversion reactions.

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Targeting enzymes of the sulfur assimilation pathway for the development of new antibiotics

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The rising trend of bacterial antibiotic resistance urges for new strategies for antibiotics development. Inactivation of enzymes involved in cysteine biosynthesis has shown promise, causing a decreased antibiotic resistance, increased susceptibility to oxidative stress and defects in biofilm formation [(1)]. Serine acetyltranferase (SAT) catalyzes the reaction between L-serine and acetyl coenzyme A to produce *O*-acetylserine (OAS). OAS is the substrate of the last enzyme in cysteine biosynthetic pathway, the pyridoxal 5'-phosphate-dependent enzyme O-acetylserine sulfhydrylase (OASS), that is expressed as two isozymes, OASS-A and OASS-B. Despite SAT playing a key role in cysteine biosynthesis, only three molecules have been reported so far as SAT inhibitors, leaving its inhibition quite unexploited. On the contrary, reversible inhibitors for both OASS isozymes were identified [(2-5)], although only active on the soluble enzymes due to their inability of crossing Gram negative bacteria membrane.
In order to identify new SAT inhibitors, several compounds, derived from in *silico* screening in house library, were tested against StSAT exploiting a newly developed spectrophotometric assay. Inhibitors with IC50 in low micromolar range were identified and proved them to be competitive against acetyl coenzyme A. The most potent inhibitors were screened in microbiological assays against *Escherichia coli* and were shown to be active only in the presence of an enhancer of membrane permeability.
In parallel, the potency as StOASS inhibitors was evaluated for i) a series of compounds, designed to be more membrane permeable, and ii) fluoroalanine derivatives potentially acting as irreversible inhibitors.

This work was supported by Marie Slodowska Curie ITN INTEGRATE project, grant number 642620. NF, JM and KG are INTEGRATE project fellows.

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GAPDH inhibition, an effective strategy against tumor proliferation

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Cancer cells largely use glycolysis to obtain both chemical energy (ATP) and metabolic intermediates for anabolic reactions and several studies proved that the blockage of the glycolytic pathway can be considered an affective anticancer strategy. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a pleiotropic glycolytic enzyme, which can represent a potential metabolic target for therapy against cancer.
Our studies focused on the anti-proliferative effects of a panel of seven newly synthesized inhibitors of GAPDH which can *in vitro* bind to the active site of the enzyme in Cystine 152. Among them we selected the best GAPDH inhibitor on the basis of its capacity to efficiently block GAPDH activity in pancreatic ductal adenocarcinoma (PDAC) cell lines, to counteract L-lactate secretion and to stimulate autophagy and apoptotic cancer cell death. Furthermore, this inhibitor, namely AXP3019, is able to synergistically inhibit PDAC cell proliferation in combination with the standard chemotherapeutic drug gemcitabine and to eradicate PDAC tumor mass in a nude mice cancer model.
Our results support the hypothesis that GAPDH inhibition alters glycolytic metabolism of cancer cells causing cancer cell mortality without affecting normal cells or inducing cytotoxic effect in mice. In conclusion, inhibition of glycolysis will serve a new approach for the identification of effective metabolic drugs, suggesting GAPDH as an effective target for anticancer therapeutic intervention.

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Biochemical and structural features of enzymes from an Antarctic metagenome

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Organisms living in constantly cold habitats have evolved different adaptive strategies including the evolution of cold-active enzymes. The latters can find applications in diverse biotechnological fields, such as detergency, food processing, transformation of heat-labile compounds [1].
Our lab is actually working on lipases, super-oxide dismutases and glycosyl hydrolases (GHs) identified from the genomic analysis of the Antarctic ciliate *Euplotes focardii* and its bacterial consortium. The aim of our work is to assess the structure and the function of these putatively cold-adapted enzymes.
More in detail, the work on GHs deals with a β-galactosidase and two putative chitinases. Amino acid sequence analyses suggest that the β-galactosidase belongs to the family of GH42 and that, beside the functional domain, it also contains a trimerization domain, and a domain with unknown function. β-galactosidase reveals activity at low temperature. Notwithstanding its origin, this enzyme exhibits also a relatively high activity at 37°C and 50°C and high number of Cys residues partially involved in disulfide bonds.
According to their amino acid sequences, the putative chitinases have been classified as GH19 enzymes, able to cleave β-1,4 glycosydic bonds in composite, chitin-like polymers. Both enzymes show lysozymatic activity against *Mycrococcus lysodeikticus* sacculi. Preliminary information is reported about the reaction rate and physical properties in different conditions.

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Superoxide dismutases from the Antarctic psychrophilic marine ciliate Euplotes focardii

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Antarctic organisms are exposed to severe stress conditions induced by low temperature and reactive oxygen species[1]. We have studied in depth three superoxide dismutases from Euplotes focardii, a ciliate isolated in Antarctica. SOD (EC 1.15.1.1) is a ubiquitous metalloenzyme that catalyzes the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide ($2O_2^{\cdot} + 2H^+ \rightarrow O_2 + H_2O_2$) [2]. The sequences of three genes encoding for SOD have been identified from a genomic study, two of these belong to the SOD1 family and coordinate Cu and Zn ions (Ef-SOD1a and Ef-SOD1b) [3], one gene encodes SOD2 (Ef-SOD2) that coordinates Mn or Fe ions. We show that the three SODs are expressed at different levels at 4°C, the physiological temperature of growth of E. focardii, and couple activity in the cold with an unusual stability at high temperature.

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Onconase cytotoxicity in melanoma cancer cells: promising results to be enforced by oligomerizing the protein

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Onconase (ONC) is a 104 residues basic protein extracted from the Rana Pipiens frog oocytes which belongs to the "pancreatic-type" ribonuclease super-family. In fact, although RNase A and ONC share less than 30% sequence identity, they display similar folds. Contrarily to most monomeric RNases, ONC evades the cytosolic RNase inhibitor and exerts a remarkable cytotoxic activity against cancer cells[1], which display more negatively membranes than normal cells.
ONC cytotoxicity has already been tested against some cancer lines and exploited in clinical trials against non-small-lung cancer and unresectable malignant mesothelioma[2].
Human melanoma, an aggressive malignancy characterized by rapidly growing incidence and high mortality rate, is resistant to radiation therapy and cytotoxic chemotherapy[3]. Hence, we evaluated here the ONC effects against melanoma registering a remarkable cytotoxic effect in the A375 melanoma cell line, while negligible in NHEM melanocytes. We also investigated with WB some aspects underlying these encouraging results.
Then, we recently found that ONC can dimerize through the 3D domain swapping mechanism and that its cytotoxicity increases against pancreatic cancer cells upon dimerization[4]. Hence, we are evaluating the ONC dimerization effect in A375 cells. Notably, native ONC can dimerize only through the swapping of its N-termini because its C-terminal ends are blocked by the C87-C104 disulfide. Considering that RNase C-swapped oligomers are generally more active than the N-swapped ones[5], we are producing some ONC mutants devoted to enhance the oligomerization tendency of the protein. This should increase the ONC cytotoxic activity and contemporarily counteract undesired side-effects.

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P44 - Oral Selected

Lignin valorization: the Lig system

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Lignin is the second most abundant natural substance in the world after cellulose: its degradation is mandatory for carbon recycling. The protobacterium *Sphingomonas paucimobilis* SYK-6 is an intriguing microorganism, producing several lignin-degrading enzymes[1].
The microorganism produces 2-pyrone-4,6-dicarboxylate from vanillic and syringic acids, a starting compound for biodegradable and high-functional polymers: in this pathway, the tetrahydrofolate (THF)-dependent *O*-demethylase LigM converts vanillic acid to protocatechuic acid (PCA). The recombinant LigM was overexpressed in *E. coli*, and its use in demethylation of vanillic acid and lignin model compounds was optimized[2]. LigM efficiently converts vanillic acid into PCA but the reaction requires a 10-fold molar excess of THF cofactor. In order to limit cofactor consumption, the plant methionine synthase MetE was also overexpressed in *E. coli* and used in combination with LigM in a cost-effective bioconversion system. Taking into account that the β-O-4-aryl ether linkages account for approximately 50% of all ether bonds in lignin, five Lig enzymes (two Cα-dehydrogenases, two β-etherases, and one glutathione lyase) from this protobacterium, were overexpressed in *E. coli*. The biochemical characterization allowed to reach the full multi-step bioconversion of a racemic mixture of the lignin linkage model compound (1-(4-hydroxy-3-methoxyphenyl)-2-(2- methoxyphenoxy)-1,3-propanediol, GGE)[3].
The combination of these Lig enzymes with additional, known ligninolitic activities (laccases and peroxidases)[1] makes it now possible to transform lignin into high value aromatic compounds, this representing a specific, effective and green alternative for lignin cleavage and valorization.

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P45

Expression, purification and preliminary characterization of an Anopheles gambiae cytosolic sulfotransferase

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The Anopheles mosquitoes are the vector of the *Plasmodium* parasite species causing Malaria in humans, and the control of the insect populations stills represents a fundamental tool to fight the Malaria global burden. The use of a limited number of molecules for the elimination of mosquitoes led to the selection of *Anopheles* strains that are resistant to available treatments. Therefore, there is an urgent need to identify molecular targets to develop insecticides based on new mechanisms of action.
Xanthurenic acid (XA) is one of the catabolites of the kynurenine pathway (KP) for tryptophan degradation in insects, and acts as a key inducer of the parasite gametogenesis in the *Plasmodium*-infected *Anopheles* female mosquitoes[1]. Moreover, XA play a role in the neutralization of the pro-oxidative potential associated with the insect hematophagous behaviour[2].
Recent studies identified a cytosolic sulfotransferase of *Bombyx mori* (bmST1) that transforms XA into xanthurenic acid 8-O-sulphate (XA-8-O-sulphate), which is the main excretion form of XA of the silkworm, behaving as a natriuretic factor[3]. At the light of the multifarious role played by XA in the *Anopheles/Plasmodium* system sustaining Malaria, and considering that the development of species-specific inhibitors of bmST1 is achievable, in the present work we identified the *Anopheles gambiae* open reading frame encoding the mosquito cytosolic sulfotransferase potentially equivalent to bmST1 (hereafter referred to as AgST). We expressed a recombinant form of AgST in *E. coli* and we purified the enzyme to homogeneity. Preliminary LC-MS based analysis revealed that the *Ag*ST enzyme catalyzes the conversion of XA to XA-8-O-sulphate, using PAPS as the sulphate donor. The AgST full enzyme characterization is ongoing.

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S11

Chemoenzymatic synthesis of α-N-glycoconjugates of biomedical interest

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The chemical synthesis of α-linked glycosides of especially *N*-acetyl-glucosamine (GlcNAc) is generally less tractable than their β-counterparts, hampering the discovery and the preparative production of inhibitors selective for α-*N*-acetyl-glucosaminidases. Stable α-linked analogues of GlcNAc glycosides hold great potential for the development of new selective therapies by stabilizing the structures of defective mutants related to genetic diseases. We report on the preparation of a novel α-thioglycoligase that can be used for the fast and efficient synthesis of α-N-acetylglucosamine-based glycosides.
Using the α-*N*-acetyl-glucosaminidase from *Clostridium perfringens* (CpGH89) as starting point, we prepared mutants in the acid/base residue Glu483 acting as α-thioglycoligases that showed remarkable synthetic efficiencies (up to 80% in 24h). To date, they represent the first biocatalyst selective for the GlcNAc moiety producing α-GlcNAc-N_3 and α-GlcNAc thioglycosides in high yields [1].
We demonstrate that α-GlcNAc-N_3 and α-GlcNAc thioglycosides can be obtained by preparative scale from α-thioglycoligases derived from CpGH89. These products could inhibit or stabilize the CpGH89 wild type, a proxy for the human NAGLU enzyme whose deficiency causes mucopolysaccharidosis IIIB.
This study lowers the barrier to the access of α-linked GlcNAc glycoside analogues significantly, and will facilitate the production of such compounds as molecular chaperones for biomedical applications.

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Purification of transglutaminase enzyme from Leishmania infantum promastigotes

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Transglutaminases (TGase) catalyze post-translational proteins modifications including lysine-glutamine isopeptide bonds formation. This activity seems ubiquitous and has been reported in *Leishmania* species[1], protozoan parasites living in sandflies gut and in macrophages of the mammalian host, causing serious infections in a large part of the world. It was reported important for parasite proliferation. One identified substrate is the virulence factor GP63, a zinc-dependent metalloprotease known also as leishmanolysin or *Leishmania* Major Surface Protease, the most represented protein in the parasite released exosomes, providing immunosuppressive and pro-parasitic action[2]. Several *Leishmania* strains genomes were sequenced but no apparent TGase-like sequences can be found. Thus we aimed to characterize and purify TGase from *L. infantum* promastigotes.
We confirmed TGase activity in *L. infantum* through *in vivo* and *in vitro* incorporation of fluorescein isothiocyanate-cadaverin into parasite proteins and a commercial microplate assay. In addition, we observed a calcium activation and a GTP millimolar inhibition of the enzymatic activity. The presence of TGase in *L. infantum* promastigotes was further confirmed by the positive immunostaining of cells found using polyclonal antibodies against a conserved peptide of human TGase 2 within the catalytic core.
Based on reported purification procedures[3] we used DEAE-Sepharose and Heparin-Sepharose chromatography obtaining a band with an apparent molecular weight of 54 kDa., which was confirmed to be a TGase by Western Blot analysis. In addition, this analysis enabled us to identify another band with an apparent molecular weight of 66 kDa.
2D electrophoresis and mass spectrometry will help us to identify a sequence useful to clone the enzyme. Testing its antigenicity might unveil if it can be useful in diagnosis or vaccine studies, as well as differences from mammal TGase could open new avenues as a possible drug target.

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How to dismantle an oxygen resistant [FeFe]-hydrogenase: a quest for the protein moiety role in H2 producing catalysts

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[FeFe]-hydrogenases catalyse the reversible inter-conversion of protons and electrons to H2 at high rates (up to 104 sec-1) and normally they are irreversibly inactivated by oxygen[1]. Nonetheless recently our group isolated a new oxygen resistant [FeFe]-hydrogenase, called CbA5H, from *Clostridium beijerinckii*[2]. The biochemical and spectroscopic features of this new protein suggest that the metal cluster sustaining the highly efficient hydrogen production of these biocatalysts, the so called H-cluster, is identical to all known enzymes of this class but specific characteristics in the protein moiety can affect a key technological property such as oxygen resistance. The protein scaffold is already known to influence in a crucial manner the properties of the H-cluster, which is not able to act as a hydrogen producing catalyst in the absence of the protein.
Here *in silico* studies and mutagenesis were combined to systematically dismantle the oxygen resistant protein CbA5H by targeting the multiple subtle differences highlighted in CbA5H *versus* the other known oxygen sensitive [FeFe]-hydrogenases: amino-acids substitution in the inner and outer sphere of the H-cluster and the different modular arrangement of the metal-binding domains involved in electron transfer, very recently reported as possible key regulators of catalysis. In parallel the same approach was applied to try and obtain a gain-of-function for oxygen resistance on oxygen-sensitive [FeFe]-hydrogenases such as CrHydA and CaHydA1, also producing chimeric variants in which the catalytic H-domain and the electron-transfer domains were shuffled between oxygen resistant and oxygen sensitive enzymes. A high-throughput colorimetric method allowed to screen for activity and oxygen resistance about 20 different variants, including point mutations, chimeras, truncated forms and combination of the above. The results hint to a complex interplay and multiple-effect of the protein moiety which is not easy to rationalize but that is very promising for the engineering of [FeFe]-hydrogenases with improved biotechnological features.

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P48 - Oral Selected

Humanized archaeal ferritin as a tool for cell targeted delivery

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Human ferritins have been extensively studied to be used as nanocarriers for diverse applications and could represent a convenient alternative for targeted delivery of anticancer drugs and imaging agents.
However, the most relevant limitation to their applications is the need for highly acidic experimental conditions during the initial steps of particle/cargo assembly, a process that could affect both drug stability and the complete reassembly of the ferritin cage. To overcome this issue the unique assembly of *Archaeoglobus fulgidus* ferritin (AfFt)[1] was genetically engineered by changing a surface exposed loop of 12 amino acids connecting B and C helices to mimic the sequence of the analogous human H-chain ferritin loop. This new chimeric protein was shown to maintain the unique, cation linked, association–dissociation properties of AfFt occurring at neutral pH values, while exhibiting the typical human H-homopolymer recognition by the transferrin receptor TfR1. The chimeric protein was confirmed to be actively and specifically internalized by HeLa cells, thus representing a unique nanotechnological tool for cell-targeted delivery of possible payloads for diagnostic or therapeutic purposes. Moreover, it was demonstrated that the 12 amino acids' loop is necessary and sufficient for binding to the transferrin receptor. The three-dimensional structure of the humanized AfFt has been obtained both as crystals by X-ray diffraction and in solution by cryo-EM[2].

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GCAP1 mutants in cone dystrophy: biochemical and biophysical analysis of the purified protein

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Cone dystrophy (COD) is an inherited autosomal dominant disease characterized by the progressive loss of central and colour vision. 14 mutations responsible for COD have been found in GUCA1A gene coding for the guanylate cyclase activating protein 1 (GCAP1), a neuronal calcium sensor protein involved in the Ca^{+2}-dependent regulation of guanylate cyclase (GC) in the retina. GCAP1 has 4 EF-hand motifs, 3 of which (EF2-4) able to bind Ca or Mg.
The only GCAP1 crystal structure available to date is the N-ter myristoylated form from chicken that shows the protein as a monomer [1]. On the contrary, we demonstrated that the human GCAP1 is a dimer, as recently discovered for its bovine homologue [2]. GCAP1 preserves the dimeric conformation either without the physiological myristoylation at the N-terminal or using a shorter construct lacking the 10 C-terminal amino acids predicted to be disordered. We used thermophoresis and size exclusion chromatography (SEC) experiments to follow the dimerization process of the wt GCAP1, estimating a dimerization constant.
SAXS analysis of *wt* protein either in batch or after SEC allowed us to produce a low resolution model of GCAP1 dimeric assembly. Moreover, SAXS analysis of the pathological D100G mutant (in EF3) shows a Kratky-Debye plot compatible with a highly flexible protein, suggesting a partial loss of its overall fold. Interestingly, some effects of D100G mutation have been observed also during a short molecular dynamic simulation (100 ns).
Finally, with the ultimate goal of partially recovering GCAP1 mutants activity, we used *in silico* docking to identify small molecules predicted to interact close to the GC/GCAP1 interface (near EF1 motif). Preliminary microscale thermophoresis data showed that one of the identified ligands is able to bind to *wt* GCAP1, and thus might represent a starting point towards the development of therapeutics against COD.

This work is supported by the Research Grant GGP16010 from the Telethon Foundation.

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Structure-function studies of mRNA localisation

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mRNA localisation is a process that regulates gene expression in a variety of organisms, developmental stages and cell types. For example, it is active to ensure timed, localised enrichment of proteins that are essential for body patterning in the fly and neuronal plasticity in mammalian cells. mRNAs are transported as complexes with regulatory proteins, most of which are RNA-binding proteins. Linear and structured signals *in cis* are required for transport, as well as RNA-associated mRNP components. An outstanding question in the field is how transcript-specificity in mRNA localisation is achieved. In particular, the recognition of double stranded RNA (dsRNA) signals is poorly understood. To address this question, we determined the structure of mRNP components required for mRNA localisation and characterised their RNA-binding properties *in vitro* and *in vivo* in the fly. Our studies show that most of RNA-binding is driven by strong non-specific interactions but that base-specific contacts are present. These contacts might confer preference for specific transcripts *in vivo*. Examples of the mode of dsRNA recognition by canonical and non-canonical RNA-binding proteins as well as perspectives for RNA specificity will be discussed.

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Electrostatic interactions drive native-like aggregation of human alanine:glyoxylate aminostransferase

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Protein aggregate formation is the basis of several misfolding diseases, including those displaying loss-of-function pathogenesis. Although aggregation is often attributed to the population of intermediates exposing hydrophobic surfaces, the contribution of electrostatic forces has recently gained attention. Here, we combined computational and *in vitro* studies to investigate the aggregation process of human peroxisomal alanine:glyoxylate aminotransferase (AGT), a pyridoxal 5'-phosphate (PLP)-dependent enzyme involved in glyoxylate detoxification[(1)]. We demonstrated that AGT is susceptible to electrostatic aggregation due to its peculiar surface charge anisotropy and that PLP binding counteracts the self-association process. The two polymorphic mutations P11L and I340M exert opposite effects. The P11L substitution enhances the aggregation tendency, probably by increasing surface charge anisotropy, while I340M plays a stabilizing role. In light of these results, we examined the effects of missense mutations leading to primary hyperoxaluria type I (PH1), a rare genetic disorder associated with abnormal calcium oxalate precipitation in the urinary tract[(2)]. We found that, under physiological conditions, the most common PH1-causing mutations endow AGT with a strong propensity to form aggregates stabilized by electrostatic forces. A similar behaviour can also occur as a consequence of mutations of surface residues altering charge distribution. Overall, we generated a global model describing the forces governing the AGT aggregation process, indicating that the contribution of electrostatic interactions in determining the fate of proteins and the effect of amino acid substitutions should not be underestimated. These findings not only increase the knowledge of the biochemical properties of AGT and of the pathogenesis of PH1, but also constitute the base for designing strategies to improve the stability of the protein as a possible premise for the development of innovative therapeutic approaches.

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NEO1: structural insights into yeast flippases

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The P-IV ATPases are eukaryotic membrane proteins whose primary role is to translocate phospholipids from the outer to the inner side of the cell membrane[1]. In humans, dysfunctions of this proteins are linked to diseases like cholestatic disease, Alzheimer's disease, obesity and type 2 diabetes. Furthermore, an imbalance of phosphatidylserine flipping is a molecular signal for apoptosis[2].
The yeast *Saccaromyces cerevisiae* expresses 5 different P-IV ATPases, four of them co-expressed with a β-subunit from the CDC50 family. With the final aim to elucidate structural details of P-IV ATPases, we carried on a general assessment of recombinant protein overexpression yields in yeast cells in different conditions (temperature, presence of metals or DMSO in the culture broth[3]), using 4 of the 5 genes coding for P-IV ATPases in *S. cerevisiae*. To easily detect the expression of P-IV ATPases in the yeast cells and quantify their presence in membranes by UV excitation, the protein was fused with GFP[4]. Since Neo1 is the only *S. cerevisiae* P-IV ATPase that does not require a β-subunit and is essential for the cell survival, we focused on finding the best chromatographic method for this protein purification and we are trying now to crystallize it and to solve its structure using X-ray diffraction.
A secondary aim of our research is the determination of the effect of single point mutations on the Neo1 protein sequence on recombinant expression, in particular mutating residues located in the phosphorylation and de-phosphorylation sites. The objective is to reduce the ATPase activity and decrease protein toxicity for the host cells and increase recombinant expression[5].

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Structural Vaccinology for a Melioidosis Vaccine: An Overview

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Structural vaccinology (SV) approaches can be used to specifically design protein immunogens, with applications in both vaccine development and immunodiagnostics[1]. SV is predicted to take a front seat in future vaccine development targeting human diseases that currently thwart therapeutic efforts[2]. The prerequisite, in addition to the 3D structure, is knowledge of neutralising epitope location. To accelerate this process, a number of in silico epitope prediction methods and epitope data repositories have evolved.
We applied a SV strategy to protein antigens from the bacterial pathogen *Burkholderia pseudomallei (Bp)* that causes melioidosis that leads to high incidences of mortality in tropical countries. By locating epitopes and synthesising them as free peptides, we demonstrated that they could acquire improved immunological properties, implying therapeutic and/or diagnostic applications[3].
Based on the availability of more than 8 *Bp* crystal structures solved by our group, and related epitope data, we are now focussing on combining multiple B- and T-cell *Bp* epitopes onto the same protein scaffold using *in silico* epitope grafting techniques[4]. This method foresees the use of computational methods to transplant immunogenic epitopes from one protein antigen onto another.
We aim to generate a chimera that induces both cell-mediated and antibody-mediated responses - essential for a melioidosis vaccine. Furthermore, knowledge of the 3D structures of chimeric antigens and resulting immunological activities will help us to correlate structure-function properties that may in turn aid successive cycles of antigen redesign.

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Expression and purification of Nfix

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Nuclear Factor 1 X (Nfix) is a transcription factor which plays an essential role in skeletal muscle[1].
The study of a mice model specifically deleted for Nfix in the post-natal life (*Nfix-null*) showed that Nfix is essential for muscular regeneration[2]. In a pathological context, precisely in a Muscular Dystrophy (MD) mice model (Sgca-null), the absence of Nfix (*Sgca-null:Nfix-null*) ameliorates the pathological features of the disease. Thus, the absence of Nfix protects from the progression of MD *in vivo*[3]. So far, MD remains incurable. This study shed light on the possible use of Nfix as a genetic tool to act on this disease.
Therefore, my project aims of solving the structure of Nfix alone and in complex with its DNA sequence, to design a structure-based drug for Nfix inhibition.
For Nfix expression we tested different protein constructs, all containing the DNA Binding Domain (DBD) and additional N-terminal/C-terminal regions. We cloned the constructs in a pMAL-cRI vector containing the Maltose Binding Protein (MBP) tag and an additional 6-Histidine tag above Nfix sequence. This allows us to use, as purification step, a Nichel-affinity chromatography and a following Size Exclusion Chromatography (SEC). So far, the protein yield obtained is about 300µg of protein for 1L of colture.
We performed biophysical analysis, such as circular dichroism and thermal denaturation suggesting a well folded native structure. Electrophoretic mobility-shift assay performed with Cy5-labeled DNA reveals that the construct can bind its target DNA and it is stable in solution. So far, crystallization tries are ongoing.
With this contribution, we report for the first time the production of Nfix and preliminary data on its biophysical characterization.

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P54

Characterization of Glycogen synthase kinase 3β and its interaction with SR90 inhibitor

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Glycogen synthase kinase 3β (GSK3β) is a serine/threonine kinase with key roles in transduction of regulatory and proliferative signals, such as insulin and Wnt signaling, synaptic plasticity, cell death, neuronal functions [1]. GSK3β is also involved in the hyperphosphorylation of protein Tau, one of the pathological hallmarks of Alzheimer's disease [2]. GSK3β inhibitors may have a role in Alzheimer's disease treatment.
The general aim of this study is the characterization of the binding mode of a GSK3β covalent inhibitor called SR90. SR90 is believed to block GSK3β activity with a mechanism that includes a covalent interaction with the kinase and a reversible ATP competition.
We expressed 3 constructs of GSK3β in insect cells, codifying for the full-length protein (1-420), and for two truncated forms (25-393 and 35-386). The shorter construct was selected as the most promising for its higher expression rate and the lack of the terminal flexible domains that may negatively influence crystallization processes [3].
With the hanging drop technique, we obtained Apo GSK3β crystals that were subsequently soaked in a solution with SR90 whereas with the sitting drop technique we obtained co-crystals of GSK3β with SR90. We collected the X-ray diffraction pattern of soaked crystals at 2.5 Å and of sitting drop co-crystals at 2.3 Å. Both structures revealed the presence of the inhibitor covalently bound to the catalytic cysteine located at the hinge region.
Protein-inhibitor binding was further investigated with Thermal Shift assay, GSK3β melting temperature was significantly increased by the inhibitor demonstrating a strong binding potency.

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Nucleotide Excision Repair and direct DNA damage reversal in Mycobacterium tuberculosis: a structural perspective

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Mycobacterium tuberculosis (MTB) is a human pathogen that during its complex life cycle is continuously exposed to a variety of DNA-damaging stresses. The mutagenic effects of O6-alkylated guanine in DNA is counteracted by the action of the suicidal O6-methylguanine methyltransferase protein (OGT). We present here the crystal structure of wild-type MtOGT in complex with a modified dsDNA, gaining structural insights into the MtOGT cooperative DNA-binding mechanism[1]. The MTB DNA repair toolbox includes also multi-enzymatic DNA repair systems such as the Nucleotide Excision Repair (NER). The first steps in NER are carried out by the coordinated action of the UvrA, UvrB and UvrC proteins. We analyzed the hydrodynamic properties and the oligomeric state of the MTB UvrB showing that the protein forms dimers in solution, which are characterized by an elongated shape, as determined by SAXS analysis[2]. Moreover, we analyzed the UvrA/UvrB lesion sensing/tracking complex by adopting a SEC-based approach followed by a SAXS-based structural analysis, revealing that the two proteins interact in solution, in the absence of ligands, with an A_2B_2 stoichiometry. Surface plasmon resonance analysis showed that the dissociation constant of the complex falls in the low micromolar range that could represent the basis for a fine modulation of the complex architecture. Finally, the characterization of such complexes in terms of thermodynamics and stoichiometry provides an experimental framework to screen molecules for their capability to interfere with the DNA-lesion searching response in mycobacterial NER.

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R180T variant of Ornithine δ-aminotransferase associated with Gyrate Atrophy: biochemical, bioinformatics and X-ray studies

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Human ornithine δ-aminotransferase (hOAT), a pyridoxal-5'-phosphate-dependent mitochondrial enzyme, catalyzes the conversion of L-ornithine (L-Orn) and α-ketoglutarate to glutamic-γ-semialdehyde, which spontaneously cyclizes to form pirroline-5-carboxylate (P5C), and glutamate[1]. hOAT deficiency is responsible for gyrate atrophy (GA), an autosomal recessive disorder characterized by progressive degeneration of the choroid and retina, leading to blindness in young adults[2]. Over 30 missense GA-causing mutations were identified so far. The aim of this work is to shed light on the structural and/or functional effects of the pathogenic R180T mutation[3]. Arg180, located at the active site, is engaged in a H-bond with the α-carboxylate of the substrate. The wild-type and R180T variant in the purified recombinant form have a similar secondary structural profile and comparable tertiary and quaternary structures. Nevertheless, the R180T mutant exhibits kcat and Km values for L-Orn about 100-fold lower and higher, respectively, than the corresponding values of the wild-type. The crystal structure of the variant was solved at a resolution of 1.8 Å. Although wild-type and mutant share a similar overall conformation, the Arg180→Thr replacement induces a relaxation of the backbone of the stretch His329-Gly320-Ser321. This shift makes the active site of the mutant sub-optimal for substrate binding, in agreement with the remarkable reduction of L-Orn binding affinity. Moreover, molecular docking of L-Orn-PLP external aldimine predicts that, unlike the wild-type, the R180T mutant can accommodate L-Orn in two alternative orientations, in which both the α- and δ-amino groups compete for covalent PLP binding and conformation of the external aldimine. Experiments are in progress to establish if the reaction of the R180T mutant with L-Orn might generate, in addition to P5C, 1-pyrroline-2-carboxylate, the product of the α-transamination reaction.

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Crystal structure of the thermophilic O6-alkylguanine-DNA alkyltransferase SsOGT-H5 in complex with a fluorescent probe

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Protein tags are powerful tools that find a huge number of applications in biotechnology and life sciences. The suicidal reaction of the human O6-alkylguanine-DNA-alkyl-transferase protein (OGT) has been exploited for the development of the SNAP-tag®, a well-known self-labeling protein tag that is mainly used in standard experimental conditions.
SsOGT-H5 protein is an engineered variant of OGT protein from the hyperthermophilic archaeon *Sulfolobus solfataricus*, representing a thermostable alternative to the SNAP-tag® technology[1]. Here we present the crystal structure of SsOGT-H[5] in complex with the fluorescent probe SNAP-Vista Green™ (SsOGT-H5-SVG) [2]. Our studies describe, for the first time, the conformation adopted by the protein after incorporating the fluorescent group in its active site. Moreover, we identify the amino acids that contribute to both the overall protein stability in the post-reaction state and the coordination of the fluorescent moiety stretching-out from the protein active site. We gain new insights regarding the conformational changes occurring to the OGTs upon reaction with modified guanine base bearing bulky adducts; indeed, our structural analysis shows an unprecedented conformational state of the protein active site loop that at is likely to trigger protein destabilization upon alkltransferase reaction. Interestingly, the fluorescent probe plays an important role in restoring the interaction between the N- and C-ter domains of the protein, which is lost following the self-labeling reaction. Molecular dynamic simulations provide further information into the dynamics of SsOGT-H[5]-SVG structure, highlighting the role of the fluorescent probe in keeping the protein stable in its post-reaction state.

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NF WHY? Structural studies on transcription factors

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The transcriptome of each living cell is ultimately determined by sequence-specific binding of transcription factors (TFs) to DNA elements within regulatory regions of genomes. NF-Y is a TF that binds the conserved CCAAT box, a regulatory element present in 30% of eukaryotic promoters, especially those of cell cycle genes. NF-Y is an heterotrimer composed of NF-YA, NF-YB and NF-YC subunits. NF-YB/NF-YC have a Histone Fold Domain related to core histones. NF-YA possesses a conserved α-helical domain involved in NF-YB/NF-YC association and sequence-specific DNA binding[1-3].
X-ray crystallography allowed us to describe the structural determinants of the NF-Y subunits, the 3D architecture of their complex, the molecular details of the DNA binding at the CCAAT box[1-3]. Small angle X-ray scattering was used, instead, to unravel the 3D architecture of higher order complexes formed between NF-Y and other TFs that act synergistically for transcription activation. Moreover, we provided the first structural insights for NF-Y in plants where, unlike in animals and fungi, a significant expansion occurred in the number of genes encoding NF-Y subunits. As a consequence, a wide variety of NF-Y trimeric complexes can form, perfectly suited to face the many environmental conditions that a plant can experience. Our structural analysis highlights similarities and differences in the plant NF-Y subunits relative to the mammalian proteins.

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PyMod 2.0: improvements in protein sequence-structure analysis and homology modeling within PyMOL

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Motivation:
The recently released PyMod GUI integrates many of the individual steps required for protein sequence-structure analysis and homology modeling within the interactive visualization capabilities of PyMOL. Here we describe the improvements introduced into the version 2.0 of PyMod.

Results:
The original code of PyMod has been completely rewritten and improved in version 2.0 to extend PyMOL with packages such as Clustal Omega, PSIPRED and CAMPO. Integration with the popular web services ESPript and WebLogo is also provided. Finally, a number of new MODELLER functionalities have also been implemented, including SALIGN, modeling of quaternary structures, DOPE scores, disulfide bond modeling and choice of heteroatoms to be included in the final model.

Availability and Implementation:
PyMod 2.0 installer packages for Windows, Linux and Mac OS X and user guides are available at http://schubert.bio.uniroma1.it/pymod/index.html. The open source code of the project is hosted at https://github.com/pymodproject/pymod.

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Biochemical and structural studies of the Drosophila TNF receptor Grindelwald

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Disruption of polarity and impairment of programmed cell death are hallmarks of advanced epithelial tumors, whose progression often involves JNK signaling. However, the link between loss of polarity and JNK signaling still remains elusive. We recently characterized a novel *Drosophila* TNF receptor, named Grindelwald (Grnd). Grnd triggers apoptosis by binding the unique fly TNF Eiger via its extracellular domain (ECD) and by recruiting on the intracellular surface Traf2, the most upstream component of the JNK pathway. Intriguingly, in *RasV12;scrib-/-* polarity-perturbed epithelia, Grnd interacts with the polarity protein Veli to promote hyper-proliferation and invasiveness in an Eiger-independent manner. These observations depict Grnd as the first TNFR able to couple cell polarity with tumor overgrowth. To shed light on the mechanism of Grnd activation by Eiger, we determined the crystal structure of Grnd-ECD, comprising a single cysteine-rich domain (CRD). The structure of Grnd-ECD consists of a β-hairpin followed by two α-helices, with an overall topology resembling the fold of human Fn14, BCMA and TACI-CRD2. To map the interacting surface between the receptor and Eiger, we engineered point mutations on Grnd-ECD and performed binding assays revealing that the β-hairpin tip and the first α-helix are the major determinants of the interaction. The K_D of the binding between the two proteins measured by Isothermal Titration Calorimetry is in the nanomolar range. To uncover the topology of the Grnd-ECD:Eiger complex, we are currently setting up crystallization experiments to obtain the crystal structure. *In vivo* assays performed using WT and mutated Grnd will address the relevance of the interaction with Eiger in inducing apoptosis in cells.

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Engineering Methionine γ–Lyase from Citrobacter freundii for anticancer activity

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Methionine deprivation of cancer cells that are deficient in methionine biosynthesis has been envisioned as a therapeutic strategy to reduce cellular viability[1,2]. Methionine γ-lyase (MGL), a tetrameric pyridoxal 5'-phosphate dependent enzyme that catalyzes the α,γ-elimination of methionine to ammonia, methanethiol and α-ketobutyrate, has been exploited to selectively remove methionine from cancer cell environment. In order to increase MGL catalytic activity, we performed sequence and structure conservation analysis of MGLs from various microorganisms. Whereas most of the residues in the active site and at the dimer interface were found to be conserved, residues located in the C-terminal flexible loop, forming a wall of the active site entry channel and possibly regulating ligand accessibility to and from the active site were found to be variable. Therefore, site-saturation mutagenesis was carried out at four independent positions of the C-terminal flexible loop, P357, V358, P360 and A366 of MGL from *Citrobacter freundii*, generating libraries that were screened for activity. We identified the variant V358Y that exhibits a 1.9-fold increase in the catalytic rate and a 3-fold increase in K_M, indicating that residues of the entry channel affect enzyme catalysis. The 3D-structure of V358Y MGL was solved to gain insight into the structural basis of the altered catalytic efficiency. V358Y cytotoxic activity was assessed towards a panel of cancer and non-malignant cell lines and found to exhibit IC_{50} lower than the wild type. In parallel, MGL was formulated in different matrices, including PLGA, silica gel, chitosan and protamine sulfate, as a strategy to increase bioavailability. The cytotoxicity activity towards a panel of cancer and nonmalignant cell lines was assessed for soluble and encapsulated MGLs. With the exception of PLGA particles, all encapsulated MGL inhibited cancer cell growth with IC50 comparable to the soluble protein. Overall, these findings indicate that encapsulation of MGL is a potentially powerful strategy for cancer cell therapy. Ongoing research activities are aimed at achieving specific and targeted delivery to tumor cells.

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Unveiling (Class III) Gene Transcription through Integrated Structural Biology

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RNA Polymerase (Pol) III is the eukaryotic nuclear enzyme devoted to the transcription of essential non-coding RNAs, including the entire pool of tRNAs, the 5S ribosomal RNA and the U6 spliceosomal RNA. Yeast Pol III comprises 17 subunit and accounts for approximately 750 kDa in mass.
Initiation of gene transcription by RNA Pol III requires the activity of TFIIIB, a complex formed by Brf1, TBP and Bdp1. TFIIIB is required for recruitment of Pol III and to promote the transition from a closed to an open Pol III pre-initiation complex (PIC), a process stimulated by the activity of the Bdp1 subunit. Here we present two cryo-EM reconstruction of an open RNA Pol III PIC at 3.8 Å and 3.3 Å, and a reconstruction of RNA Pol III at 3.0 Å.
The structures presented unravel the molecular mechanisms underlying RNA Pol III transcription initiation, highlighting the specific features of this highly efficient essential machinery but also the general conserved mechanisms of gene transcription initiation.
We also present the crystal structures of a vertebrate specific TFIIIB complex, containing the Brf2 subunit, a protein overexpressed in lung and breast cancers. Brf2 encompasses a redox-sensing switch, capable of turning on and off transcription of target genes in a redox-dependent manner. Integrating structural, biochemical and functional data in living cells we discovered Brf2 to act as a master switch of the oxidative stress response and established a mechanistic link between Brf2-dependent Pol III transcription and cancer.

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UK114 is a member of the conserved Rid protein family of imine deaminases involved in preventing metabolic stress

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Metabolism is characterized by a complex network of integrated biochemical pathways that could also lead to the production and accumulation of reactive metabolites with potential damaging effects on cellular components. An example is 2-aminoacrylate (2AA), a reactive and toxic enamine generated by amino acid metabolism and interconverted in the tautomer 2-iminopyruvate. These intermediates are produced by pyridoxal phosphate (PLP)-dependent serine/threonine dehydratases or by cysteine desulfhydrases[1].
Recently, a family of enzymes named Reactive intermediate deaminase (Rid) with a role in preventing the accumulation of reactive enamines/imines was discovered. Rid protein family is conserved in all domains of life and comprises eight subfamilies. The severe defects generated by the absence of RidA demonstrate the importance of these enzymes in buffering 2AA stress.
UK114, the proposed mammalian member of the RidA subfamily, was identified as a component of perchloric acid-soluble extracts from goat liver. It exhibited immunomodulatory properties, but its function is still unclear. This work addressed the question of whether goat UK114 is a Rid enzyme. Biochemical analyses demonstrated that recombinant UK114 hydrolyzes α-imino acids generated *in vitro* by L- or D-amino acid oxidases and the specificity of UK114 toward different imino acids was determined2. Circular Dichroism (CD) analyses highlighted the remarkable conformational stability of UK114. The half-life of heat inactivation at 95 °C, measured from CD and activity data, was about 3.5 h[2].
In conclusion, mammalian UK114 proteins are highly stable RidA enzymes that may play an important role in metabolism homeostasis.

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Session V · Other/No theme allocated

P61

Structural and biochemical studies of potential selective inhibitors of the aldehyde dehydrogenase 1A1 and 1A3

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Aldehyde dehydrogenases (ALDHs) are NAD^+-dependent enzymes capable of oxidizing aldehydes to the respective carboxylic acids. In mammals, 19 genes encoding different ALDHs have been identified, located on different chromosomes [1]. Recent evidences highlight a direct correlation between the overexpression of ALDHs enzymes in cancer stem cells to a marked resistance against chemotherapy and radiotherapy related with an increase malignity of tumors. An altered ALDH1A1 expression has been detected in non-small cell lung cancer in response to cyclophosphamide treatment [2]. Similarly, it has been observed that an increase of the ALDH1A3 expression level in mesenchymal glioma steam cells is strictly correlated with a pharmacoresistance to temozolomide in glioblastoma [3].
Our research is focused on the structural and biochemical study of the two isoenzymes ALDH1A1 and ALDH1A3. With the aim of developing potent and selective inhibitors towards both isoforms, we have expressed and purified to homogeneity the two ALDHs isoforms, and a library of structure-based, rationally-designed compounds was tested [4, 5]. All compounds were initially screened at a fixed concentration of 25 μM for both the two isoforms, leading to the identification of four potent and selective compounds towards both isoenzymes, and the respective IC50 values were subsequently calculated. Crystallographic studies are currently underway on both enzymes in order to determine the two isoforms structure in complex with selected compounds and to elucidate the molecular determinants of the potency and specificity of the inhibitors.

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Alteration of the catalytic behaviour and of the conformational stability of phosphoglycerate kinase 1 natural variants

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Phosphoglycerate kinase 1 (PGK1) is an ubiquitous enzyme expressed in all somatic cells that catalyzes the reversible phosphotransfer reaction from 1,3-bisphosphoglycerate to ADP. Recently it has been discovered to be overexpressed in many cancer types. Cancer cells acquire an unusual glycolytic behaviour reprogramming their cellular metabolism in a way that confers an evolutionary and thermodynamic advantage. Furthermore, under hypoxic conditions they shift from oxidative phosphorylation to aerobic glycolysis, a behavior known as Warburg effect.
Emerging evidences underline noncanonical protein kinase functions for PGK1, suggesting that it can be considered as a moonlighting metabolic enzyme in tumor development making it promising therapeutic target for cancer.
Several somatic variants of PGK1 have been identified in tumors as nonsynonymous single nucleotide polymorphisms (nsSNPs) that occur in the coding region leading to amino acid substitutions in the protein sequence. In this study we chose from COSMIC database (http://cancer.sanger.ac.uk/cosmic) some nsSNP variants of PGK1 and we analyzed the effect of the amino acid substitutions on the PGK1 structure and stability and on the enzymatic function. The variants display a decreased catalytic efficiency and/or thermodynamic stability and an altered local tertiary structure. The changes in the catalytic properties and in the stability of the PGK1 variants, mainly due to the local changes evidenced by the X-ray structures, suggest also changes in the functional role of PGK1 to support the biosynthetic need of the growing and proliferating tumour cells.

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Insight into the structural and functional characterization of the anserinase from Oreochromis niloticus

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Carnosine, anserine and homocarnosine are the three most representative compounds of the histidine dipeptides, widely distributed in mammals in different amounts depending on the species and the tissue considered[1]. Histidine dipeptides are mainly degraded by two different carnosinase homologues: a non-specific cytosolic form (CN2) and a highly specific metal-ion dependent carnosinase (CN1) located in serum and brain. The hydrolysis of imidazole-related dipeptides in prokaryotes and eukaryotes is also catalyzed by aminoacyl-histidine dipeptidases like PepD, PepV and anserinase (ANSN). ANSN is able to degrade anserine (β-alanyl-1-methylhistidine) and shows a broad specificity[1]. It has been postulated that CN1 and ANSN are paralogs derived from the most recent common ancestor in vertebrates indeed carnosinase resembles anserinase in hydrolytic ability against carnosine, anserine and homocarnosine. Such naturally occurring dipeptides represent an interesting topic because they seem to have numerous biological roles such as potential neuroprotective and neurotransmitter functions in the brain. ANSN results to be a very interesting target of study either from an evolutionistic point of view and for its localization in the brain, retina and vitreous body of all vertebrates containing N-acetyl-histidine in their tissues[2]. In the present study, we report, for the first time, cloning, expression in *Escherichia coli*, purification and a preliminary characterization of the ANSN from the fish *Oreochromis niloticus*. Furthermore ANSN has been expressed in an eukaryotic system in order to perform a functional comparative study. Finally, by means of a mass spectrometry-based proteomic approach, we got insight into the structural features of the protein sequence and post-translational modifications.

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The Protein Facility of Elettra: a new platform to support research and development

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A Protein Facility has been recently implemented at the Elettra synchrotron of Trieste taking advantage of the infrastructures and of the expertise existing in the structural biology laboratory of Elettra. The protein production platform is organized for expression in *E.coli*, insect and mammalian cell systems, and is well equipped to go from small-scale constructs screening to mid/large-scale preparations, with strong capacity and competence in protein purification techniques. On average we support about 30 different protein projects a year coming from internal and external users of academic or industrial R&D groups. In particular the lab intent is to facilitate the process to obtain recombinant proteins suitable for analysis with synchrotron radiation techniques (x-ray diffraction, SAXS, FTIR). The lab is also opened to less experienced researchers such as young postdocs and PhD students offering tutoring and knowledge transfer. The active projects are distinguished between collaborative research, industrial services and tutoring activities.
We have a more profound expertise on studying protein targets for drug discovery with specific interests for two big proteins families of druggable targets: the human kinases and the deubiquitinases that are relevant in cancer and neurodegenerative diseases. In addition, our pipeline encompasses between different types of proteins such as membrane proteins, proteases as well as nanobodies. The Facility is an active member of the P4EU Network (Protein Production and Purification Partnership in Europe; www.p4eu.org) contributing also in benchmarking studies[1] that allow technology advancements and maintenance of the state-of-the-art in the protein production field.
The present poster is intended to introduce the protein production facility of Elettra Sincrotrone Trieste to the scientific community, showing the major achievements and future developments of the facility in the field, drawing from a panel of the most relevant results.

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AUTHORS' INDEX

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