**Homology Modeling and Structural Analyses of Serotonin Receptors**

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Serotonin is a G-protein coupled receptor (GPCR) which is synthesized in the serotonergic neurons of the central nervous system and the enterochromaffin cells of the gastrointestinal tract. Serotonin receptors have been studied extensively for its involvement in treatment of psychological disorders such as depression, anxiety, obsessive compulsive disorder and obesity [1]. Objective of this study is to use a structure-based approach to assist in designing potent compounds for monoamine receptor subtype 5HT1A. Absence of serotonin class crystal structure necessitates the usage of existing crystal structures as templates for homology modeling. Due to its sequence similarity, Rhodopsin based β2 adregenic receptor is vastly used as template for modeling GPCR proteins. Recently, there have been five new high resolution structures of β2 adrenergic receptor, which improved the model building process of 5HT1A. The Schrödinger suite was used for comparative modeling of 5HT1A and its structural analysis. Homology models were built using prime and the docking was performed using flexible docking method known as Induced Fit Docking (IFD). We considered as a test set consists of benzeindole derivatives, which act as 5HT1A agonist. In agreement with mutational experiments, Asp116, Ser199, Thr200, and Asn386 were observed as key ligand binding residues [2]. Preliminary results for Glide, Energy Model and IFD scores correlate inconsistently with respect to experimental $K_i$ values of benzeindole derivatives requiring further refinement of the existing model.

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**In-house Characterization of Proteins using X-ray Powder Diffraction**

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X-ray powder diffraction (XRPD) on in-house sources can be successfully used for identification of polymorphs of proteins [1]. Thereby it offers a convenient and quick way to characterize polymorphs and substrate-protein complexes in the laboratory. As the need for fast characterization of proteins is growing, the technique may find applications in the enzyme production industry as well as in the pharmaceutical industry for characterization of drug-candidates, which more often are proteins themselves [2]. Growing suitable protein crystals of a certain size and quality is one of the major bottlenecks in solving protein structures from single-crystal X-ray diffraction experiments. To be able to screen early precipitates for crystals by in-house XRPD will save large amounts of time and efforts.

In this study XRPD patterns for lysozyme and different crystalline polymorphs of insulin were recorded on a standard in-house powder diffractometer [1,3]. Collecting in-house protein powder diffraction data requires careful handling of the samples. Specially designed sample holders combined with optimized collimation were found to be the key factors in improving the data quality and reducing the data collection time.

For identification the experimental patterns were compared with patterns calculated from known crystal structures. Very good agreement with Protein Data Bank (PDB) data was obtained after (a) including straightforward corrections for background, (b) determination and optimisation of unit cell parameters from the experimental pattern, and (c) including the contribution to the scattering from disordered bulk-solvent in the calculated pattern. The solvent correction was found crucial for a good agreement, as the solvent typically occupies 30–70 % of the volume in a protein crystal. The bulk-solvent was modelled by the flat bulk-solvent model [4] as a constant level of electron density in the voids between the macromolecule and the contribution was added to the calculated structure factors. The correction procedures were demonstrated by identification of three different conformations of hexameric insulin.

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## Predicting thermostability: Xylanases as experimental model system

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The in-house computer software PROPKA was used to predict amino acid mutations that could lead to an increase in the thermal stability of the glycosyl hydrolase family 11 (GH11) *Bacillus circulans* xylanase (BCX). PROPKA (http://propka.ki.ku.dk) is a computer program that can calculate and display the pKa values and the pH-dependent contribution to the free energy on unfolding for ionizable groups in proteins[1,2]. A recent study showed that the calculated overall stability correlates well with the experimentally observed thermal stability data for wild type BCX[1]. In this study we have used PROPKA to calculate and compare the stability contributions for homologous residues in several GH11 xylanases, thereby indentifying stabilizing motifs found examined xylanases but not present in highly similar mesophilic BCX[3]. These motifs (N25R/N181D among others) have been introduced in BCX by site directed mutagenesis with the expectation that they would increase the thermal stability. These BCX variants were expressed, purified and analyzed for increased thermal stability by CD and the stability was compared to wild type protein in terms of $T_m$. The N25R/N181D variant appears, in contrast to the prediction, very similar to wild type. We are determining the structure of the variant by X-ray crystallography, in order to establish whether the salt bridge the mutation aims to introduce is indeed formed and provide feedback for improvement of PROPKA predictions.

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## Local Moves for efficient sampling of protein conformational space

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During their function proteins often undergo local conformational rearrangements. Standard molecular dynamics simulations cannot address the typical time scale at which these processes occur ($10^{-6}$ -$10^{-3}$ s). Monte Carlo simulation is an alternative methodology to compute pathways and thermodynamic properties of proteins. To probe the conformational space around the densely packed native state a method to perform local deformations of protein backbone is required.
We present a novel method to perform local deformations of protein backbone that includes an analytical solution for the loop closure problem. We show that this kinetic enables reliable and efficient sampling of protein equilibrium.

**Structural Biology of Transcription Factor CI from TP901-1 by Crystallography and Small Angle Scattering**

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The molecular mechanisms that determine the expression of one or another set of genes resulting in different development pathways have been studied intensively in bacteriophage. Temperate phages, as opposed to virulent virus, may choose to enter either a lytic or a lysogenic lifestyle following infection of a sensitive host. In the lytic infection cycle, new phages are produced, followed by lysis of the host cell and liberation of phage progeny into the surroundings, whereas in the lysogenic infection cycle, the phages genom typically integrates into the bacterial chromosomal DNA, resulting in a dormant prophage, mainly expressing genes required for maintenance of the lysogenic state. The decision to enter either the lytic or the lysogenic life cycle is controlled by a bistable genetic switch consisting of phage promoters responsible for transcription of genes required for either the lytic or lysogenic growth, including phage-encoded protein regulating these promoters. One of the main players involved in the regulation of the two life cycles is a phage-encoded repressor protein, CI, which represses transcription from the lytic promoter $P_L$ by binding to multiple operator sites on the DNA[1,2]. In this project we aim to characterize CI as well as various constructs of CI and the CI-DNA complex using crystallography and small-angle scattering techniques, to obtain further understanding of the mechanism of repression.

Various construct of CI were cloned, expressed in E.coli and purified. Subsequently, crystals were obtained however only for one of the construct namely the C-terminal domain of CI (CTD-CI). CTD-CI is responsible for the oligomeratation of the presumed hexamer of CI complex. Because of little sequence homology with known structures SAD/MAD techniques have been applied to overcome the phase-problem. Crystal was soaked in NaBr and X-ray data was recorded. Because of X-ray damage only the peak dataset was recorded a resolution ~2.2 Å the declined diffraction ability caused lower resolution data for inflection and remote (~2.7 Å). Despite processing performed with XDS indicates anomalous scattering of brome-derivate dataset, the phase-problem still has to be overcome.

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# Crystallographic studies of a protein:DNA complex at (too?) low resolution

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As a model system for protein:DNA interactions, we study the NAC transcription factors[1], in particular ANAC019 from Arabidopsis. The structure of the NAC domain alone has been solved previously (PDBID: 1UT4). We have obtained low resolution (4.5Å) crystallographic data on the complex with a 26 bp nucleotide as well as both model phases and experimental phases. However, the resulting electron density maps are of very low quality and not suitable for interpretation in terms of atomic positions. This is due to both the low resolution and inaccurate phase information. Many computational procedures exist to optimize data processing and improve the quality of the maps (density modification). These have been extensively sampled but so far, the major breakthrough has not come.
In addition, we have obtained small angle X-ray scattering data of good quality. Ab initio modeling support the crystal data. However, an in-depth analysis of the size of the particles suffers from the lack of accurate (and easily accessible) quantification of protein and DNA in the sample.

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**Structure of NADPH-dependent Thioredoxin Reductase from Barley**

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Thioredoxins (Trx's) are protein disulfide reductases that regulate the intracellular redox environment. Furthermore they participate in the regulation of a large number of cellular processes including DNA synthesis, oxidative stress response and apoptosis. They are present in several isoforms in plants of which the cytosolic Trx h isoforms are reactivated by specific NADPH-dependant thioredoxin reductases (NTRs) [1].

NTRs catalyze the reduction of a redox-active disulfide bond in Trx using NADPH as reductant and FAD as cofactor. NTR consists of two domains, the FAD and the NADPH binding domains, and can shift between two conformations; the flavin oxidizing (FO) or the flavin reducing (FR) conformation. To obtain the latter, one domain has to rotate 66° relative to the other. Only by covalent binding of NTR to Trx can the FR be stabilized for characterization [2].

We have solved the structure of one isoform of NTR from barley (HvNTR2) which is closest to the FO conformation [3]. Based on this structure a model of the FR conformation in complex with a Trx from barley (HvtrxH2) has been made and will be evaluated by site-directed mutagenesis. Furthermore we would like to find methods for measuring the shift from one conformation to the other as well as the binding between NTR and Trx.

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**Structure-function relationship of fungal β-1,4-galactanases: pH dependence of thermostability and activity**

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Endo-β-1,4-galactanases hydrolyze the β-1,4-O-glycosidic bonds in both galactan and arabinogalactan, which are a component of the "hairy" part of pectin. They are used as industrial enzymes for example for processing of fruit and vegetable juices and wine. Endo β-1,4-galactanases are assigned to glycoside hydrolase Family 53 (GH-53) (http://www.cazy.org/).

Currently, we are using fungal galactanases as model system to test our ability to manipulate the pH and temperature dependence of stability and kinetic properties of industrial enzymes based on crystal structures. We have previously determined the structure and characterized the pH and temperature dependence of three fungal β-1,4-galactanases, AAGAL [1] (from *Aspergillus aculeatus)*, HIGAL [2] (from *Humicola insolens*) and MTGAL [2] (from *Mycelyophtora thermophila*), and now extend our structural studies to include MGGAL (from *Meripleus giganteus*).

Here we present the current work on determining the pH dependent thermostability by differential scanning calorimetry of native AAGAL and MTGAL and comparing this to the thermostability predicted by PROPKA [3] on the basis of the known 3D-structure. Furthermore we present the kinetic characterization AAGAL activity on a polymeric substrate, giving insight into the behavior of the enzyme as function of pH, which is complicated by substrate inhibition.

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# Can soy tolerance protect against peanut allergy?

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**We have**
an animal model for food allergy. This model has been used for studying the sensitization potential of related food allergens; 7S globulins from peanut (Ara h 1), soy (Gly m 5), hazelnut (Cor a 11) and pea (Pis s 1).

**Methods**
7S globulins were purified from their 'normal' matrix (raw or mildly processed foods) by extraction of crude proteins followed by gel permeation chromatography. Brown Norway (BN) rats were bred and kept on two different diets; a 'maize flour' diet contaminated with 1-25 µg soy/g diet (soy-tolerant rats) and a 'rice flour' diet without soy (non-tolerant rats).
Rats were immunised intraperitoneally with 100 µg of purified Ara h 1 or Gly m 5 per rat without the use of adjuvant. Rat sera obtained at sacrifice were analysed for 7S-specific IgG1 and IgE responses by ELISA. Functionality of specific IgE was examined by Rat Basophilic Leukaemia (RBL) cell assay.

**Results**
Sensitisation of soy-tolerant or non-tolerant rats with Ara h 1 induced comparable level of IgG1 and IgE when measured by ELISA. In contrast, sera from non-tolerant rats immunised with Ara h 1 showed a statistically higher degranulation in RBL assay and stronger IgG1 binding in inhibition ELISA compared to sera from soy-tolerant rats. Soy tolerance also influenced the ability of Ara h 1-specific antibodies to bind to related 7S globulins as well as the functionality and binding capacity of the Cor a 11- and Pis s 1-specific antibody response although to a less degree.

**Conclusion**
Although the four 7S globulins are related they induced antibodies with different antigen binding characteristics. In addition soy tolerance influenced the function of antibodies to Ara h 1.
This study indicates that *inherent protein properties such as glycosylation, tertiary structure and amino acids displayed on the protein surface may be decisive for allergenicity* of the protein. This finding is new. There is no knowledge of which structural features characterises a food allergen.

**We need**
help to look at ***protein structure and surface characteristics to help understand why closely related proteins induce qualitatively different immune responses.***

# Wound healing effect on tissue composition -Facing interindividual variability

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The project is initiated as part of an overall investigation on how the quality of fish produced in aquaculture is influenced by the way the fish is handled both before and after slaughter.

Rainbow trout is the most important fish in aquaculture in Denmark and of significant commercial interest. Recently it has become increasingly clear that the entire life and sickness history of the fish potentially can influence quality. Tissue damage is a naturally occurring phenomenon in fish grown in aquaculture, and can occur as a consequence of mechanical damage like handling, interaction with other fish or contact with its surroundings.

The experimental design included 7 fish that were uninjured and 7 fish that were injured according to the model. The fish used for the experiment were one year old rainbow trout with an average weight of 640 g and an average length of 34 cm. Fish were kept at 15 °C in 200-l tanks at the aquarium facilities at Division of Industrial Food Research. At start of the experiment 7 fish were anaesthetized and injured. Sampling was performed at day seven and including one sample from each uninjured fish and two samples (one from within the injured area and an internal control from the uninjured area) from each injured fish. Protein expression profiles of the samples were achieved by 2-dimensional gel electrophoresis (coomassie staining). In this way protein spots with an apparent range of molecular masses from 10 to 120 kDa and p*I* values from 4 to 7 were separated. Tandem mass spectrometry is used to identify protein spots of interest.

**High throughput analysis of human immunoregulatory proteins by flow cytometry**

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Plasmacytoid dendritic cells (pDCs) have been associated with both beneficial and detrimental effects during an HIV-infection. Little is presently known about the intracellular pathways in pDCs that mediate these processes, but evidence suggest that C-type lectin receptor (CLR) crosstalk with Toll-like receptors (TLR) may play an important role. The present objective was to study the CLR-TLR crosstalk that is induced in human pDCs upon challenge with HIV-derived TLR and CLR ligands. We used 8-color high throughput flow cytometry for the analysis of pDC surface proteins with immunoregulating properties.

We tested three different recombinant HIV-1 gp120s: IIIB (CHO-derived), 96ZM651 (Sf9-derived) and BaL (HEK-293-derived) which mediated diverse modulation of TLR7-induced IFN-alpha-2a and CD86 expression in pDCs. These differences may possibly be explained by different posttranslational glycosylations in the different gp120-expression systems. The three gp120s all lead to inhibition of TLR7-mediated CD40-upregulation and increased CCR7-upregulation. The modulating effects of gp120 were concentration-dependent and no significant modulations were obtained below concentrations of 100 μg/mL. Finally we have found that TLR7-activated pDCs express the mannose receptor (CD206), which may play an important role in the observed CLR-TLR crosstalk.

# Is Protein:Protein Interaction Involved in Regulation of the Genetic Switch of Phage TP901-1?

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The genetic switch of the temperate lactococcal phage TP901-1 controlling the choice between the lytic or lysogenic life cycle is regulated by two phage encoded proteins, CI and MOR, transcribed from two divergently oriented promoters. The CI protein represses transcription from both the lytic and the lysogenic promoter while MOR seems to work as an anti-repressor at the lytic promoter, and simultaneously, as a co-repressor at the lysogenic promoter (1,2,3). The *cI* gene encodes a protein of 180 amino acids that binds sequence specific to three operator sites, $O_R$, $O_L$, and $O_D$ (2). The N-terminal domain of the CI protein contains a Helix-Turn-Helix DNA-binding site whereas the C-terminal domain contains the oligomerization domain (4,5).
The *mor* gene encodes a protein of 72 amino acids that contains a putative Helix-turn-Helix motif although the MOR protein not by itself represses transcription of either promoters *in vivo* (3). However, in the presence of CI transcription, the lysogenic promoter may be tightly repressed. It is suggested that a protein complex consisting of CI and MOR is required for this repression and that the ratio of CI:MOR determines the state of the lysogenic promoter. We are working on verifying the protein:protein interaction between CI and MOR and want to determine the stoichiometry of such a complex. We are very interested in determining the relative ratio between the two proteins both *in vivo* and after co-purification of these proteins *in vitro*.

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# NNF-Center for Protein Research; the Protein Production Unit

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The Protein Production Unit is dedicated to the production, purification and characterisation of proteins of medical relevance using both bacterial and eukaryotic expression systems.
The goal of the Protein Production Unit is to recombinantly generate as many soluble and active human proteins as possible for use by our collaborators in their research. We have currently set up a high-throughput *E.coli*-based cloning, expression and purification facility where we process 300-400 constructs every month for cloning and small scale expression screening. From these, 30-40 protein domains are purified each month to a double digit mg scale and are characterised using SDS-PAGE and mass spectrometry.
Additionally, we are currently setting up eukaryotic cell based high-throughput systems (using mammalian or insect cells) through which we will process ~100 constructs a month.
To achieve these goals, we are upgrading both our expression and the purification capacity to be able to handle larger scale intra and extracellular expressions from both *E.coli* and the eukaryotic cell systems.

The Protein Production Unit produces purified proteins both for internal CPR projects and for external scientific collaborations. We are engaged in developing new high-throughput cloning and expression technologies to improve the generation of solubly expressed recombinant proteins. In addition, collaborations within the field of recombinant protein expression are of interest to us; in particular the development of library based technologies for *in vivo* screening and selection of solubly expressed proteins.

# EXPLORING XYLANASE ACTIVITY AND ITS ENDOGENOUS INHIBITORS IN BARLEY GRAINS

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Barley grains *(Hordeum vulgare)* in general are genetically diverse and their composition varies with cultivar and significantly affected by environmental, climatic and agronomic factors (1-2). The mature barley grain is intensively used as feed and contains a battery of hydrolytic enzymes that are synthesized during seed development and deposited in an inactive form in the grain. These enzyme activities (also referred to as first wave enzymes) are of fundamental biological significance as they are required for initiating seed germination, moreover they are considered important for maximizing nutrients digestibility in food and feed. Deep knowledge is lacking on the particular enzyme forms and occurrence constituting such first wave enzymes, including β-amylases, phytases, proteases, lipases, and cell wall degrading enzymes e.g. β-glucanases and xylanases (3-6). Given their potential for improvement of food and feed quality, surprisingly little is known about the composition, synthesis, deposition, activation and biochemical properties of these enzymes. The present project sets out to unravel the full complement of these first wave enzymes and identify and characterize their biochemical and enzymatic properties. As an initiative, the variability of xylanase (microbial vs. plant) and xylanase inhibition activities in different barley cultivars have been determined. Preliminary results suggest that there is a considerable intercultivar variation in the level of microbial xylanases as well as of endogenous xylanases. Inhibition assays are in progress and will provide detailed information about the level and ratio of xylanase and its inhibitors in barley grains.

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# The *Lactococcus lactis* thioredoxin system

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*L. lactis* is a gram-positive micro-aerophilic bacterium producing lactic acid as its primary catabolic end product. The resulting acidification is explored in industrial fermentations where *L. lactis* is used in starter cultures for production of cheese and buttermilk. In the presence of oxygen, *L lactis* is exposed to oxidative stress caused by reactive oxygen species (hydrogen peroxide, superoxide anion radical and hydroxyl radical). Responses to oxidative stress are regulated by various mechanisms among which the thioredoxin (Trx) system plays important roles. Trx contains a redox-active CXXC motif, reduces disulfide bonds in target proteins, and receives reducing equivalents from the NADPH-dependent flavoprotein thioredoxin reductase. Many organisms also rely on the glutathione/glutaredoxin system for disulfide reduction but *L. lactis* lack glutathione biosynthetic apparatus and it can thus be suggested that the thioredoxin system is of particular importance in this organism. *L. lactis* contains an NADPH-dependent thioredoxin reductase (TrxB), two thioredoxins (TrxA, TrxH) and a glutaredoxin-like protein NrdH. The latter one works as source of reducing power for NrdEF ribonucleotide reductase (Jordan *et al.*, 1996). It has similar fold a glutaredoxin, but it is reduced by TrxB. TrxA is similar to the "classical" thioredoxin found in organisms ranging from bacteria to higher eukaryotes with a CGPC active site motif. TrxH, however, display a divergent sequence (14% identity and 30% similarity to TrxA) and contains an atypical CGDC motif that appears to be restricted to gram-positive bacteria. To study the *in vivo* functions of TrxA and TrxH in *L. lactis*, knockout mutants have been constructed and the phenotypes analyzed. The results so far indicate that TrxA is involved in oxidative stress response while the role of TrxH remains unclear.

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**Coordinated redox regulation of transferases involved in starch biosynthesis in *Arabidopsis thaliana***

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Important structural and catalytic functions of many enzymes are dependent on regulation determined by the redox state of the cell. This regulation occurs through the breaking and reformation of disulfide bonds of the target proteins and has been described especially for many chloroplastic enzymes. In the chloroplast, reducing equivalents produced during the day by photosynthesis are transported from photosystem I via the ferredoxin-thioredoxin system to the target proteins. Hence, this system links enzyme activity to light, ensuring coordination between photosynthesis and metabolism by reductive activation of enzymes during the day.

Some specific redox regulated enzymes active in starch metabolism have been identified in *Arabidopsis*. These include the ADPglucose pyrophosphorylase, the beta-amylase BAM1 and the starch phosphorylator GWD1, all of which are reductively activated in vitro.

Using an activity screen based on manipulation of redox potentials in *Arabidopsis* extracts we have identified a number of redox regulated degradative and biosynthetic enzymes involved in starch metabolism. Subsequent characterization of specific enzyme activities has confirmed this regulation. Our data specifically demonstrates that two isoforms of the starch synthases, SS1 and SS3, and one isoform of the branching enzymes, BE2, are reductively activated. Our data demonstrate evidence for the presence of a coordinated regulative mechanism of starch biosynthesis providing simultaneous coordination of the multitude of enzymes responsible for structuring the starch granule and its close link with the flow of photosynthetically fixed carbon into starch.

**Protein core facility:**

**Amino acid analysis, protein sequencing and mass spectrometry**

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Enzyme and Protein Chemistry, Department of Systems Biology, building 224, DTU, hosts the protein core facility – for more detailed information and guidelines:
see link http://www.epc.bio.dtu.dk/English/Protein%20core%20facility.aspx

The protein core facility offers:

- Amino acid analysis
  - Amount
  - Composition and identification
- Protein identification
  - Identification of proteins
  - Homology search
  - Information of post-translational cleavage
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- Mass spectrometry
  - Identifications of proteins

The services of the core facility are available for researchers and students at DTU, other academia and commercial customers.

For more practical information: You are welcome to contact us at the Protein.DTU workshop.

# Quantifying the Evolution of Function in *Sperm-whale* Myoglobin

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The function of myoglobin (Mb) an oxygen binding protein in muscle cells is quantified and evaluated in terms of two ratios (oxygen storage ratio and oxygen transport ratio) and two indices (oxygen storage index and oxygen transport index). These descriptors are measured at four different cellular oxygen pressures of $P_{O_2}$=40 ,20,10 and 5 mmHg to cover the whole physiological range from normoxic to anoxic conditions. The significance of Mb is larger in terms of all descriptors at lower cellular oxygen pressures. Using a 90-mutant data set of sperm-whale Mb, it is shown that the wild-type structure has been positively selected in special residual sites mainly in the first shell of the protein around the heme group. Moreover, the vast majority of amino acid replacements in residues far from heme is found to be nearly neutral in terms of function. The distribution of functional proficiency among mutants is left-skewed, representing the effect of negative mutations in the whole population. The evolutionary preference of the wild-type protein is also investigated from a structure-function relationship. For amino acid replacements that are chemically similar, the function is not changed significantly while for dissimilar replacements, mutations can impair the function to a great extend. It is anticipated that results can be used to quantify the molecular evolution of Mb and similar ligand-binding proteins and employed for rational engineering of carrier proteins.

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**Analysis of early events in the interaction between *Fusarium graminearum* and the susceptible barley (*Hordeum vulgare*) cultivar Scarlett**

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**Abstract**

A proteomic analysis was conducted to map the events during the initial stages of the interaction between the fungal pathogen *Fusarium graminearum* and the susceptible barley cultivar Scarlett. Quantification of fungal DNA demonstrated a sharp increase in fungal biomass in barley spikelets at three days after inoculation. This coincided with the appearance of discrete *F. graminearum* -induced proteolytic fragments of β-amylase. Based on these results, analysis of grain proteome changes prior to extensive proteolysis enabled identification of barley proteins responding early to infection by the fungus. In total, the intensity of 51 protein spots was significantly changed in *F. graminearum* -infected spikelets and all but one were identified. These included pathogenesis-related proteins, proteins involved in energy metabolism, secondary metabolism and protein synthesis. A single fungal protein of unknown function was identified. Quantitative real-time RT-PCR analysis of selected genes showed a correlation between high gene expression and detection of the corresponding proteins. Fungal genes encoding alkaline protease and endothiapepsin were expressed during 1-3 days after inoculation, making them candidates for generation of the observed β-amylase fragments. These fragments have potential to be developed as proteome-level markers for fungal infection that are also informative about grain protein quality.

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