BENZON SYMPOSIUM No. 50

THE LIPOCALIN PROTEIN SUPERFAMILY

AUGUST 24-28, 2003, COPENHAGEN, DENMARK

Organizing committee:
Bo Åkerström (Lund), Darren Flower (Compton), Jean-Philippe Salier (Rouen) and Niels Borregaard (Copenhagen)

Abstracts - MONDAY, August 25, 2003

Structural Relationships Between Bacterial Lipocalins and Outer Membrane Beta-barrel Proteins
Bishop RE, University of Toronto, Toronto, Ontario, Canada
Lipocalins represent a class of mostly soluble 8-stranded beta-barrel proteins that generally bind hydrophobic ligands. However, the most ancient lipocalins are often bound to the outer membranes of Gram-negative bacteria by a lipid anchor. Among the closest eukaryotic relatives of the bacterial lipocalins, ApoD and Lazarillo are also found to be peripherally anchored in membrane environments. Interestingly, integral membrane proteins of bacterial outer membranes also adopt a beta-barrel architecture that can range in size from 8 to 22 beta-strands. A formal resemblance to the lipocalins is seen in the two 8-stranded outer membrane beta-barrels known as OmpA and OmpX. Aside from an absence of detectable amino acid sequence similarity, the small transmembrane beta-barrels are contrasted from the lipocalins by a more extended barrel architecture, a smaller shear number, and, importantly, a mostly polar barrel interior that is occluded with solvent molecules. Our collaborative studies into the structure and function of an integral outer membrane enzyme have now identified a small transmembrane beta-barrel with an even closer resemblance to lipocalins. PagP is an enzyme that functions to transfer a palmitate chain from a phospholipid to the lipid A anchor of lipopolysaccharide in the outer membrane. Insights into the structure and dynamics of PagP in detergent micelles has been obtained using NMR spectroscopy and x-ray crystallography. PagP is an 8-stranded beta-barrel preceded by an N-terminal amphipathic alpha-helix. Unlike most transmembrane beta-barrels, the upper half of PagP, exposed to lipid A in the outer leaflet of the membrane, exhibits several conserved proline residues that disrupt the continuity of hydrogen-bonding between certain beta-strands. Additionally, the 1.9 Angstrom resolution crystal structure reveals a detergent molecule bound in the upper half of the barrel interior that identified a hydrophobic palmitate binding pocket at the active site. These findings imply that PagP allows access of membrane lipids through the beta-strands to the barrel interior. The possibility that lipocalins were adapted from a protein that is similar to PagP will be discussed.

Tear lipocalin genes – Structures and regulatory elements
Redl B, Department of Molecular Biology, Innsbruck, Austria
Due to a lack of the third lipocalin consensus motif Tear lipocalins (also called VEGs) were classified as "outlier lipocalins". However, their structure and genomic organisation very closely resemble those of the genes encoding "kerneal lipocalins". All of them consist of six protein-coding exons and a 3´-nontranslated exon, with a size of exons and an intron phasing very similar to other lipocalins. When comparing Tear lipocalin genes from various species a very high conservation is found. Interestingly, in all species investigated, more than one copy of these genes is present, but not all of these copies seem to be expressed under normal conditions. Since Tear lipocalins are expressed in various organs, tissues and cells, either in an inducible or constitutive mode, we have analyzed some of the promoters.

There are significant differences in the presence of regulatory elements, e.g. human lipocalin genes contain regulatory elements, such as AP-1 and AP-2 sites, NF-kB sites, and cAMP-responsive elements, which are absent in pig. However, most noteworthy, in all of the promoters analyzed we found metal responsive elements (MRE), which have been described to be specific for metallothionein genes, so far.

Analysis of the promoter regions was the basis for further investigations which significantly enlarged our knowledge of the tissue expression and physiological activity of tear lipocalins, in general.

Chromosomal location and organization of lipocalin genes
Salier JP[1], Benhadj A[2] & Risler, JL[2], [1]Inserm Unit 519, Faculty of Medicine-Pharmacy, Rouen, France, and [2] CNRS UMR 8116, Genome and Informatics**,** Evry, France
Lipocalins with their extreme diversity at the primary sequence level and their unity at the 3D structure and gene arrangement levels form a paradigm for a superfamily. The number of lipocalin genes seems to have significantly expended from plants to animals and from insects to vertebrates and it culminates in mammals. Two conserved

features of the lipocalin genes in mammals are a shared location at a limited number of chromosomal areas as well as limited variations from a canonical exon/intron arrangement (exon number and size; intron phase). As protein sequence similarity can hardly be used to identify distant paralogs as novel members of the lipocalin superfamily, we have relied upon some of the exon/intron features mentionned above as a query to search for as yet undescribed lipocalin genes in the human and mouse genomes. This search was done in the curated gene sets of the ENSEMBL data bank (http://www.ensembl.org/). Although the genes listed within ENSEMBL do not exhaustively cover the above genomes, all known lipocalin genes were retrieved. A limited number of further candidate genes with as yet no association with the lipocalin superfamily were found. They were checked for the presence of some lipocalin hallmarks, such as the mandatory GxW(F/H/Y/W/basic) motif in the corresponding protein sequence, as well as appropriate exon and protein sizes and a location at an expected chromosomal area. On such grounds, none of the candidate genes could be reliably considered as a *bona fide* lipocalin-encoding gene. This suggests that our current "draft of the lipocalin genome" in mammals is close to completion.

Molecular evolution of the lipocalin family

Sánchez D[1], Ganfornia MD[1], Gutiérrez G[2], and Marin A[2]. [1]IBGM, Universidad de Valladolid-CSIC, Valladolid, Spain. [2]Departamento de Genética, Universidad de Sevilla, Sevilla, Spain

Lipocalins are currently confirmed to be present in bacteria, protoctists, plants, arthropods and chordates. We have assessed the molecular evolution of lipocalins by inferring amino acid sequence and gene structure phylogenies. Protein phylogenies suggest an evolutionary scenario for the family where bacterial lipocalins were inherited by unicellular eukaryotes, and passed onto plants and metazoans. Metazoans then spread a low number of lipocalins into their successors. The primitive arthropod and chordate lipocalins were likely similar to the Lazarillo and ApoD lipocalins. Alongside the chordate radiation, the ApoD-like ancestral lipocalin duplicated and gave rise to the ancestor of RBPs, and to a set of paralogous lipocalins that subsequently diversified.

Intron position and phase is well preserved among lipocalin clades. We have used intron features to reconstruct a phylogeny by maximum parsimony and distance methods. We have also analyzed the variability of introns present in the C-termini of lipocalins, and compare lipocalin intron arrangement with tertiary structure. The phylogeny based on intron arrangement shows that metazoan lipocalins have more introns, and that introns have been gained at the C-termini of chordate lipocalins. Also, we have built a consensus tree based on protein sequence and gene structure phylogenies. The congruence of phylogenetic trees built from these two independent sets of characters increases the verisimilitude of the lipocalins history reconstructed from both of them.

Finally, we are currently identifying the tree determinants, the molecular elements that, according to our phylogenies, could account for the functional diversification of lipocalins. This will help us design experiments to test the contribution of specific residues or sequence segments to each particular lipocalin function

Poster No. I-1

Cold-regulated plant lipocalins

Charron, J-B F, Hecheima M & Sarhan F, Département des Sciences Biologiques, Université du Québec à Montréal, Montréal, QC, Canada

Lipocalins are a large and diverse group of small, mostly extracellular proteins implicated in many important functions such as modulation of cell growth and metabolism, binding of cell-surface receptors, membrane biogenesis and repair, induction of apoptosis and environmental stress response. Two novel lipocalin family members were identified from wheat and *Arabidopsis*. They were designated TIL (temperature-induced lipocalin) and CHL (chloroplastic lipocalin). Northern analyses demonstrated that *til* transcripts are upregulated during cold acclimation, heat-shock, hight-light, and rose-bengal treatments while *chl* transcripts are specifically upregulated by cold acclimation. Structure analyses indicated the presence in both TIL and CHL of the three structurally conserved regions that characterize lipocalins. Sequence analyses revealed that TILs share homology with three evolutionarily related lipocalins: the mammalian apolipoprotein D, the bacterial lipocalin and the insect Lazarillo protein. The comparison of the putative tertiary-structures between the human apolipoprotein D and the wheat TIL suggests that the two proteins differ in membrane attachment and ligand interaction. Transient expression of TIL-GFP fusion in onion epidermis cells showed that the protein accumulates at the plasma membrane. The putative functions of these novel plant lipocalin members during cold acclimation and oxidative stress are discussed.

Poster No. I-2

The chondrogenesis associated lipocalins

Pagano A[(1,2)], Giannoni P[(2)], Randazzo N[(2)], Zerega B[(2)], Zambotti A[(2)], Sanchez D[(3)], Gutierrez G[(4)], Ganfornina MD[(3)], Descalzi-Cancedda F[(5)], Cancedda R[(1,2)] & Dozin B[(2)], [(1)] Dipartimento di Oncologia Biologia e Genetica, Università di Genova., [(2)] Istituto Nazionale per la Ricerca sul Cancro, Genova. [(3)] IBGM, Univ of Valladolid-Spain, [(4)] Dept. Genetics, Univ de Sevilla, Spain; [(5)]Consiglio Nazionale delle Ricerche, IBFM, Genova, Italy

Endochondral ossification in chicken was studied in order to identify new stage-specific molecular markers we isolated and cloned three genes (and a pseudogene) located in the same chromosomal locus (Ex-FABP, CAL β, CAL

γ, CAL δ). The structure of the cluster is concordant with an origin based on tandemly repeated duplication events by unequal crossing-over during meiosis. Tissue expression analysis evidenced a very similar pattern for all the members of the cluster with liver as a very important site of transcription. In situ hybridization, immunohistochemistry and Quantitative Real-Time RT-PCR experiments showed that all the members of the cluster are highly expressed in hypertrophic fully mature chondrocytes while only a barely detectable signal is observed in undifferentiated and proliferating cells. This indicates that these proteins are specific for the hypertrophic stage of the cartilage during long bone formation. We also demonstrated a strong increase of gene expression for all the cluster elements after inflammatory stimuli. Experiments on in vitro cultured chondrocytes indicated that the cluster is highly expressed also in quiescent cells. Our evolutionary analysis shows that CAL γ is the ortholog of the human PGD2 Synthase and that CAL δ resembles the complement C8γ lipocalin further evidencing the existence of an ancestral cluster from which mammals and sauropods lipocalins have derived. We thus propose that CALs are a cluster of lipocalins expressed in stress conditions acting co-ordinately and synergistically in different biological processes.

Poster No. I-3

Identification of a cluster of epididymal lipocalins on the murine chromosome 2 [A3] region

Suzuki K, Lareyre J-J, Araki Y, Orgebin-Crist M-C & Matusik RJ, Vanderbilt University Medical Center, Nashville, TN, USA

We have shown that the epithelium of the mid/distal caput epididymidis secretes an androgen-dependent epididymal retinoic acid binding protein (mE-RABP) in the lumen. We have recently found another gene related to the mE-RABP gene 1.7 kb upstream from the mE-RABP gene. The new gene, mouse epididymal protein of 17 kDa (mEP17), is specifically expressed in the initial segment and its expression is dependent on testicular factor(s). Analysis of the mE-RABP and mEP17 gene structure revealed that these two genes belong to the lipocalin family which binds small hydrophobic molecules such as retinoids. Both genes are localized on murine chromosome 2 [A3], a region known as a lipocalin gene rich area. To identify other lipocalin genes, we have searched the mouse genome sequence database from the Celera discovery system. We found two novel epididymal lipocalin genes designated mEP19 (mouse epididymal protein 19 kDa) and mMUP4-L (mouse major urinary protein 4-like). The full-length cDNAs corresponding to each gene were obtained by RT-PCR. The mEP19 and mMUP4-L genes were specifically expressed in the epididymis. *In situ* hybridization revealed that spatial gene expression was restricted to the initial segment (mMUP4-L) and the initial segment and part of segment 2 (mEP19) of the caput epididymidis. Both genes were regulated by testicular factor(s). Interestingly, mEP19 gene was also regulated by androgens since testosterone replacement restored mEP19 gene expression only partially after castration. A computer analysis of the human genome shows that the epididymal lipocalin cluster has been conserved in human. In conclusion, the presence of a cluster of epididymal lipocalins with different spatial expression and regulation suggests that these duplicated genes have gained non-redundant physiological functions during evolution. (Supported by NIH HD36900 and the Rockefeller/Ernst Schering Foundation.)

Poster No. I-4

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is Upregulated in Human Epithelial Cells by IL-1β but not by TNF-α

Cowland JB, Sørensen OE, Sehested M & Borregaard N, Granulocyte Research Laboratory, Department of Hematology, Rigshospitalet 93.2.2, Copenhagen, Denmark

Neutrophil gelatinase-associated lipocalin (NGAL) is a siderophore-binding antimicrobial protein synthesized by human neutrophils and epithelial cells. In this work, we demonstrate by immunohistochemical staining that NGAL synthesis increases dramatically in bronchial epithelial cells and alveolear type II pneumocytes during lung inflammation. The pro-inflammatory cytokine IL-1β induced a more than 10-fold upregulation of NGAL expression in the type II pneumocyte-derived cell line A549 cells whereas TNF-α, IL-6, and LPS had no effect on NGAL expression. Similar IL-1β-selectivity was demonstrated in primary bronchial epithelial cells and epidermal keratinocytes and for a NGAL promoter fragment transfected into A549 cells. A 30 bp region (-183 to -153) of the NGAL promoter containing an NF-κB consensus site was required for IL-1β-upregulation. In both A549 cells and keratinocytes, IL-1β-induction of the NGAL promoter was completely abolished by mutation of the NF-κB site and severely reduced by a dominant negative inhibitor of the NF-κB pathway. TNF-a-activation of NF-κB did, on the other hand, not increase NGAL synthesis. Selectivity for the IL-1-pathway was substantiated by demonstrating that NGAL promoter activity could be induced by LPS-stimulation of A549 cells transiently expressing Toll-Like Receptor 4, which utilize the same intracellular signaling pathway as the IL-1 receptor. This induction was also abolished by a point mutation of the NGAL promoters NF-κB site. Together, this demonstrates a selective upregulation of NGAL by the IL-1 pathway, which is dependent upon NF-κB activation.

Heme- and Radical Savenger Properties of A_1- Microglobulin

Åkerström B[1], Allhorn M[1], Lundqvist K[2], Schmidtchen A[2], Nordberg J[1,2], Olsson ML[2], Winterbourn CC[3] & Kettle AJ[3], Department of Cell and Molecular Biology, Lund University, Sweden[1], Blood Center, Lund, Sweden[2], Department of Pathology, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand[3]

α_1-Microglobulin (α_1m) is a yellow-brown 26 kDa protein, widespread in plasma and tissues. α_1M belongs to the lipocalins, a protein superfamily with highly conserved 3D-structures, forming an internal hydrophobic ligand-binding pocket. The colour is due to heterogeneous modifications on three lysyl and one cysteinyl residue located around the opening of the lipocalin pocket. A truncated α_1m species, t-α_1m, which lacks the C-terminal tetrapeptide, LIPR, is formed when α_1m is exposed to hemoglobin. The processed t-α_1m binds heme and the t-α_1m-heme complex shows a time-dependent spectral rearrangement suggestive of degradation of heme concomitantly with formation of the heterogeneous yellow-brown chromophores associated with the protein. Normal urine contains both t-α_1m and full-length α_1m, suggesting that t-α_1m is formed in vivo. α_1M is a major heme-binding protein of ulcer fluids and is processed into the t-α_1m form. New results show that α_1m is involved in a catalytic redox-reaction with the 2,2'-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS)-radical, producing both the reduced form, free ABTS, and an oxidized purple product. Both are formed rapidly and the oxidized ABTS is bound tightly to the protein. Experiments with α_1m-mutants lacking the lysyl and cysteinyl residues around the lipocalin pocket entrance indicate that these residues are involved in the binding to oxidized ABTS. In conclusion, our results suggest that α_1m is a heme-degrading and radical-dismutating agent in the tissue compartment of the body. Heme and other low molecular weight radical molecular species are important contributors to the oxidative stress encountered after extravascular hemolysis as well as in chronic ulcer inflammation and α_1m appears to be involved in the defense.

The Structure and Function of Tear Lipocalin

Glasgow BJ, Gasymov OK, Abduragimov AR & Yusifov TN, University of California, Los Angeles, CA, USA

Tear lipocalin (TL) accounts for about one third of the protein in human tears and is expressed in lacrimal gland, Von Ebner's gland, nasal and tracheal mucosa, eccrine sweat glands, pituitary, and prostate. TL is a promiscuous lipocalin and binds a broad array of small hydrophobic molecules including phospholipids, fatty acids, cholesterol, glycolipids, fatty alcohols, retinol, tocopherol, phthalates, and lipid products of inflammation. TL exhibits endonuclease activity and inhibits cysteine proteinases. These characteristics suggest multiple lipid transport, scavenger and enzyme functions. As the principal lipid protein in tears, TL interacts with lipids to protect the ocular surface. The modulation of lipid binding depends on features such as the conserved disulfide bond, structural rigidity, and interactions of hydrophobic residue clusters as well as local environmental pH. Recent advances in spectroscopic methodologies have permitted the resolution of solution structure features of tear lipocalin. Site directed tryptophan fluorescence enabled the use of homology modeling despite the low sequence identity of TL with other lipocalins. The data characterize specific motifs that may account for some of the unique functional properties of this lipocalin.

Poster No. I-5

Lipocalins in marine mammals

Keith EO, Pervaiz S & Brew K, Oceanographic Center, Nova Southeastern University, Dania Bearch, FL. USA

The lipocalin beta lactoglobulin has been purified from the milks of a number of marine mammals, and both complete and partial sequences have been obtained. Using a variety of phylogenetic reconstruction methods several evolutionary controversies have been addressed: (1) whether the pinnipedia are monophyletic or diphyletic, (2) the nearest extant terrestrial relative of the pinnipedia, and (3) the relatedness of the walrus (*Odobenus rosmarus*), and the Odobenidae, to the two other pinniped families, i.e. the Otariidae and the Phocidae. Our results support the monophyly of the Pinnipedia, suggest that the Mustelidae are the closest extant terrestrial relative, and perhaps the ancestor group, of the pinnipedia, and suggest a relationship between the walrus and the Phocidae. Additionally our results indicate a connection between a member of the Sirenia, the Florida manatee (*Trichechus manatus latirostris*), and the Perissodactyla. These results also support the close affinity of the cetacea to the artiodactyla as determined by studies using other milk proteins.

The analysis is complicated by the fact that the bottlenose dolphin (*Tursiops truncatus*), and some other terrestrial mammals, posses two forms of the protein, which segregate to different parts of the phylogenetic tree, indicating that gene duplication likely occurred prior to species divergence. The phylogenetic distribution of beta lactoglobulin in the milks of some, but not all, mammals parallels patterns of placentation, and suggests a function of this protein in the transfer of passive immunity from mother to nursing neonate.

The use of milk proteins in phylogenetic analysis is attractive because they can be obtained with minimal trauma to the individual animal, are relatively easy to purify, and can be obtained in quantities sufficient for sequencing.

Poster No. I-6

Regulation of Rabbit Tear Lipocalin by Sex Hormones

Zylberberg C, Brew K, Kota S, Ponomareva O & Azzarolo AM, Biomedical Science, FAU, Boca Raton, FL, USA

Tear lipocalin is a member of the lipocalin family. The exact biological function is not yet established, but it is believed to carry lipids to the outer surface of the eye to prevent tear evaporation.

The concentration of tear lipocalin has been found to decrease in the tears of patients with dry eye disease. Homologues of tear lipocalin have been found in different species. A 20 kDa protein with lipocalin signature motif has been characterized in rabbit lacrimal fluid by N-terminal sequencing. The purpose of this study was to investigate the expression levels of the 20 kDa lipocalin in rabbits of different ages and gender.

Pilocarpine-stimulated lacrimal fluid was collected from juvenile and sexually mature male and female New Zealand white rabbits and was analyzed by SDS-PAGE electrophoresis and Western Blot analysis using anti-human tear lipocalin antibody. Quantitation of the bands was performed by densitometry.

The results from the densitometric analysis showed that the lacrimal fluid from sexually mature female had significantly greater expression of lipocalin than the sexually mature male animals. Also, the sexually mature rabbits had higher expression compared to the juvenile male and female. However no significant differences in expression of lipocalin was found between the juvenile male and female rabbits. The expression at the transcriptional level in the lacrimal gland was confirmed by RT-PCR.

These experiments suggest that the expression of the 20 kDa lipocalin is regulated by sex hormones. Further studies will be needed to identify the hormones and their mechanisms of action.

Poster No. I-7

Odorant/Pheromone-Binding Lipocalin Genes of Syrian Hamster

Srikantan S, Parekh VP & De PK, Centre for Cellular & Molecular Biology, Hyderabad, India

Three odorant/pheromone-binding lipocalins displaying different tissue- and sex-specific expression have been so far identified in saliva, tears, urine or vaginal discharge of Syrian hamsters. Among these, the genes for salivary **m**ale-**s**pecific **p**rotein (MSP; present also in urine and female tears) and the immunologically related **f**emale **l**acrimal **p**rotein (FLP; present in tears), shared >90 % sequence identity. MSP and FLP genes (both 5.53 kb long) had no significant match with the gene for hamster aphrodisin (present in vaginal discharge and female saliva), although aphrodisin gene was also 5.53 kb long and had similar genomic organization. MSP/FLP-like genes and their proteins (in salivary and lacrimal gland) were detected in three out of six different hamster species investigated, suggesting closer evolutionary relatedness among these three species. Sequence analysis identified rat **o**dorant-**b**inding **p**rotein (OBP)-1/1F gene (expressed in nasal mucosa of both sexes) and its putative mouse counterpart, as closest known homologues of MSP and FLP. Protein sequence analysis of lipocalins, revealed presence of a CXXXC motif only in MSP, FLP, aphrodisin, their putative homologues in rat and mouse, OBP-1a/1b of mouse nasal gland and probasins of rat and mouse prostate. A search of the sequenced genomes revealed that all the above rat and mouse lipocalin genes are located on X-chromosome, suggesting a X-chromosomal localization for MSP, FLP and aphrodisin in hamster. MSP and FLP genes have distinct differences in regulation but both display an unusual transcriptional repression by estro- gens and androgens, which was found to be mediated by respective sex-hormone receptors. However, no consensus sex-hormone-receptor binding sites were observed within a considerable stretch of their upstream sequences. Our results suggest that MSP and FLP are recently duplicated genes, which are evolutionarily closer to rat and mouse-OBP genes than with aphrodisin and in contrast to their rat/mouse homologues, MSP/FLP genes have acquired sex-hormonal regulation and different tissue-specificities.

Poster No. I-8

Embryonic expression profile of chicken Apolipoprotein D

Sánchez D[1], Pagano P[3], Tonachini L[3], Descalzi-Cancedda F[3], Martínez S[2] & Ganfornina MD[1]. [1]IBGM, U. of Valladolid, Spain, [2]Instituto de Neurociencia, Alicante, Spain, [3] Istituto Nazionale Ricerca sul Cancro/Centro Biotecnologie Avanzate, Genova, Italy

We are interested in lipocalin function during nervous system (NS) development. Mouse ApoD is the closest vertebrate relative to the arthropodan lipocalins expressed in the developing NS (the grasshopper and fly Laz genes). Phylogenetic analyses of the lipocalin family predicted the presence of a Laz-ApoD gene in birds. We have confirmed the presence of ApoD in chicken, and analyzed its expression pattern during embryogenesis by in situ hybridization (IH) and Real-Time Quantitative RT-PCR (qRT-PCR).

In the NS, chicken ApoD mRNA is first detected by IH in a subset of neurons and glia at 18 days of embryogenesis (HH stage 44), a pattern similar to the one observed in postnatal mice. However, the early ApoD expression in mouse (in meningeal precursors and NS pericytes) is absent in chicken (tested at 4, 8 and 10 days of development). Apo D is also strongly expressed in glandular epithelium associated to feather buds in the chicken skin at embryonic day 17.

Quantitation of gene expression by qRT-PCR was normalized with respect to the tissue with the lowest expression at each stage. At HH stage 39 ApoD transcript is restricted to the skin with a signal 246.48 ± 38.44 fold more

aboundant that in heart. A barely detectable signal was also observed in tibia, brain, and muscle. At HH stage 44, a strong signal (123.7 ± 11.79) appears in the brain in addition to the skin (317.2 ± 30.46). Expression in tibia, muscle, stomach, liver, heart, and stern do not differ significantly from the intestine expression (the lowest at this stage). These results support our hypothesis that neuronal and glial expression is an ancestral character in chordate lipocalins evolution. However, they also show interesting divergences in expression patterns that open the potential for early functional differentiation, a key to the remarkable diversification observed in this protein family.

Poster No. I-9

Mouse Major Urinary Proteins (MUPs) as a Pheromone Messengers: Interstrain Differences in *Mup* Gene(s) Expression is Due to Animal Physiological Status, Sex and Age

Novikov S, Churakov G, Philimonenko A, Burkot I & Morozov V, I.P. Pavlov Institute of Physiology of the Russian Academy of Sciences, St. Petersburg, Russia

There is rapidly growing evidence that the functional activity of androgen dependent pheromones in *Mus musculus L*. is strongly associated with major urinary proteins (MUPs) - typical member of the lipocalin superfamily (Flower, 1996; Novotny et al., 1999; Cavaggioni, Mucignat-Caretta, 2000; Timm et al., 2001; Sharrow et al., 2002). Thus the detailed studies of structural basis of ligand binding properties to MUPs can shed lights on the functional role of these proteins in individual "fingerprinting" (Churakov et al., 1992; Evershed et al., 1993; Robertson et al., 1996; Hurst et al., 2001). The MUPs profiles of the urine collected from female and male mice of CBA/LacY, C57BL/6JY inbred strains and their F1 hybrids were studied using PAGE electrophoresis and DEAE chromatography. Quantitative differences in expression of 7 main MUP bands were due to animal genotype, plasma testosterone level, developmental stage and sex. Castration and subsequent testosterone treatment dramatically alters the content and proportion of MUP's; this alteration was also genotype dependent. The obtained results are discussed in lights of reports on: a) genotype dependent differential *Mup* gene expression (Chr. 4), b) abundant MUP mRNA content in nasal tissues (Utsumi et al., 1999), c) the existence of several subtypes of odorant-binding proteins (OBPs) (Pes, Pelosi, 1995; Löbel et al., 1998; Tegoni et al., 2000), d) the existence of membrane receptor for OBP (Boudjelal et al., 1996). Taken together these data suggested that MUPs, OBPs and their artificial protein analogs may serve as a perspective molecular vectors for noninvasive drug targeting to the brain via transnasal delivery (Novikov et al., 2002; Novikov, 2003).

Supported by Russian Foundation for Basic Research (project 02-04-49273).

Poster No. I-10

Bacterial expression of lipocalins for functional and structural studies

Daniel Breustedt & Arne Skerra, Institute of Biological Chemistry, Technical University Munich, Freising-Weihenstephan, Germany

Although our understanding of the lipocalin family has clearly improved in recent years, many aspects, like the nature of ligand-binding or interaction with cell surface receptors, remain unclear for many of its members. Deeper insight into these relationships might come from systematic functional and structural analysis.

Here, the expression of a broad set of lipocalins in the pASK75 expression system [1] was investigated with the goal to produce highly pure protein for biochemical characterization. For this purpose, the structural genes encoding a_1-microglobulin, hNGAL, prostaglandin D synthase, tear lipocalin, complement component C8g, and the bacterial lipocalin from *E. coli* were amplified via PCR and cloned on the vector pASK75strepII. Secretion of these proteins into the periplasm of *E. coli* was achieved by fusion of the bacterial OmpA signal sequence to the amino-terminus. The resulting plasmids also encode the *Strep*-tag II affinity peptide at the carboxy-terminus of each lipocalin for simplified purification via streptavidin affinity chromatography.

We have been successful in producing all these lipocalins as soluble proteins in *E. coli*, and the one-step chromatography yielded protein of high purity as judged by SDS PAGE. Expression levels varied from 0.1 to 2.5 mg purified protein per 1 liter of bacterial culture. Determination of the ligand-binding constants has thus become possible. Therefore, our secretory expression strategy, which was first successfully applied to the retinol-binding protein [2], seems to be useful for the bacterial production of functional lipocalins in general.

[1] Skerra, A. (1994) *Gene* **151**, 131-135.
[2] Müller, H. N. & Skerra, A. (1993) *J. Mol. Biol* **230**, 725-732.

Poster No. I-11

Expression Pattern, Regulation, Characterization and cDNA Cloning of Two Odorant-Binding Tear Lipocalins of Female Hamsters

Srikantan S, Paliwal A, Stephano AQ* & De PK, Centre for Cellular & Molecular Biology, India.*Universidad Autonoma De Aguascalientes, Mexico

We identified a **m**ale-**s**pecific **p**rotein (MSP), in hamster **s**ub**m**andibular **g**land (SMG), saliva and urine, which is a lipocalin and binds odorants. Now, we report a different but immunorelated lipocalin, **f**emale **l**acrimal **p**rotein (FLP), which is female-specifically expressed in **l**acrimal **g**land (LG) and tears of adults. Interestingly, MSP is also female-

specifically expressed in LG and tears along with FLP, with a hormonal regulation different from that in SMG. FLP was however undetectable in SMG. In adult LG, both FLP and MSP display an unusual repression by androgens, estrogens and thyroid hormones. Their expression in females was due to incomplete repression by endogenous estrogens. Thus, these LG lipo- calins were maximally expressed (>20% of gland proteins) in ovariectomized, lactating, light-deprived or hypophysectomized females (all low-estrogen states) and similar levels were induced in orchid- ectomized or hypophysectomized males. Strikingly, unlike adults, FLP's but not MSP's expression in immatures was induced by androgens and its androgen repression in males was seen only, at and after puberty. Mature FLP (156 aa) and MSP (157 aa) shared 95 and 86 % identity in cDNA and amino acid sequences and MSP but not FLP, had a N-glycosylation site. FLP/MSP had maximum sequence identity with **o**dorant/pheromone-**b**inding **p**roteins such as OBP-1/1F of rat nasal mucosa (58%) and aphrodisin of hamster vaginal discharge (39%) which are lipocalins. Immunological crossreaction and significant cDNA match was only with rat OBP and no crossreaction was detected in LG/SMG of rat and mouse or in nasal mucosa of hamster. However, of the 14 amino acids, lining ligand-binding pocket of aphrodisin, 12 are identical or conserved in FLP/MSP, suggesting that FLP, like aphrodisin and MSP, might also bind odorants. Since, these female-specific LG/tear lipocalins contact the nasal epithelium and are also voided externally, they might have a role in female hamsters in perception/dissemination of odor cues.

Poster No. I-12

Sip24 in differentiation and inflammation

Ulivi V[1,2], Tutolo G[1,2], Descalzi Cancedda F[1,3] and Cancedda R[1,2], Istituto Nazionale per la Ricerca sul Cancro IST[1], Universita' di Genova[2], Consiglio Nazionale delle Ricerche I.B.F.M.[3], Genova, Italy

Sip24 is a mouse secreted lipocalin produced by quiescent Balb/c 3T3 cells and inducible by many factors, including serum, FGF-2, prostaglandin F2a and dexamethasone. It is also inducible by LPS in macrophages and is an acute phase protein expressed in liver during the acute phase response (APR) induced by turpentine injection in mouse. Sip24 induces apoptosis through an autocrine pathway in leukocytes but not other cell types. Purpose of our study is to investigate the function of sip24 in differentiation and inflammation and identify the signaling pathway leading to the expression of the protein. We have investigated sip24 expression in mouse embryos. By immunohistochemistry we observed localization of the protein in the growth plate cartilage, in the forming skeletal muscle fibers and in the developing myocardium. A mouse chondrocyte cell line, MC615, barely expresses sip24 in subconfluent cultures and strongly expresses the protein in hyperconfluent cultures when they produce type II collagen and form nodules. In subconfluent cultures sip24 expression is strongly induced by inflammatory stimuli such as LPS and IL-1 and is repressed by non-steroidal antiinflammatory drugs; the protein expressed in hyperconfluent cultures is not over-induced by inflammatory agents or repressed by non-steroidal antiinflammatory drugs.The signaling pathway leading to the expression of sip24 involves p38 MAPK whose activation is induced by both LPS and IL-1 in a time dipendent manner in subconfluent cultures; hyperconfluent MC615 cells exhibit high level of p38 activity not overinduced by treatment with inflammatory agents. Pretreatment of subconfluent cultures with SB203580 leads to inactivation of p38 and inhibition of sip24 induced by LPS. In addition pretreatment of subconfluent cultures with PMA before induction with LPS leads to PKC activation, p38 inhibition and inhibition of sip24 synthesis, suggesting a role of PKC in p38 activity modulation. Both SB203580 and PMA do not have any effect in hyperconfluent cultures. Cyclooxigenase-2 expression is involved. Measurement of NFkB activation shows a strong activation in hyperconfluent cultures.

Poster No. I-13

Identification of a lipocalin from an unsequenced genome using mass spectrometry

Turton M, Hayter J, Robertson D, Hurst J[*], Beynon R, Protein Function Group, [*]Animal Behaviour Group, Faculty of Vet Science, University of Liverpool, Liverpool, UK

The success of cross-species proteomic identification of lipocalins using peptide mass identification techniques is limited due to the low sequence conservation of this superfamily (~20%). In this study *de novo* peptide sequencing by tandem mass spectrometry (MS/MS) was used to identify a urinary lipocalin from male bank voles, *Clethrionomys glareolus*, a rodent species for which there is very limited genomic information.

Urinary lipocalins are highly expressed in some rodent species and are involved in chemical communication, binding and releasing semiochemicals. *C.glareolus* exhibited sexual dimorphic expression of urinary proteins; electrospray mass spectrometry of male samples identified a single major species at 16930 ± 2 Da. There was no evidence of individual polymorphism of this species from MALDI-TOF MS peptide analysis of wild or inbred animals as described previously with the mouse.

MS/MS characterisation of tryptic peptides from the 16930 Da species identified the conserved N terminal motif (..GXW..) of the lipocalin superfamily. Further MS/MS analysis extended the N-terminal sequence for 30 residues, revealing similarity to aphrodisin, a lipocalin from female hamster vaginal discharge. A tight intrachain disulphide loop that is highly conserved in the pheromone binding proteins, a distinct group of lipocalins, was identified by comparison of native and carbamidomethylated tryptic digests. A mass shift of 116 Da was observed between a modified and native peptide, corresponding to 2 carbamidomethylated cysteine residues (114 Da) and the reduction of an internal disulphide loop (2 Da). MS/MS analysis of the carbamidomethylated peptide confirmed this motif.

The homology of the *C.glareolus* urinary lipocalin with the pheromone binding proteins but lack of polymorphism indicates an alternative role for chemical signalling to that observed in murine species.

Poster No. I-14

Probing the molecular basis of the binding properties of rMUP mutants

Venturelli MB, Ferrari E, Casali E, Tsay A*, Chapman MD* & Spisni A, Dept. Experimental Medicine University of Parma, Italy; *INDOOR Biotechnologies Inc., VA, USA

The mouse Major Urinary Proteins (MUPs) define a class of lipocalins involved in pheromonal signaling and that, known as Mus m 1 urinary allergen complex (1,2), present immunological properties.

Objective: this study consists in the analysis of a recombinant urinary protein (rMUP) mutated with the aim of determining the conformational features critical for its ligand binding properties and immunoreactivity. *Methods:* the structural analysis of the mutants has been carried out by circular dichroism spectroscopy; binding properties were investigated by fluorescence spectroscopy using *N*-phenyl-naphtylamine (NPN, ref. 3), a specific probe of the hydrophobic calyx of rMUP; immunological assessment of mutant reactivity was performed using a specific, rabbit polyclonal Ab based ELISA for rMus m 1. This assay was highly sensitive (200pg/ml Mus m 1) and specific for mouse lipocalin.

Results: the data indicate that 1) the mutant (C138S) is a more stable variant of rMUP with binding parameters and a reactivity with polyclonal antibodies similar to the original rMUP. However, differently from the native form, it exhibits a complete refolding after thermal denaturation; 2) two variants (Y120F, Y120A), where the mutations involve the "cavity", show that the progressive loss of structural stability parallels the reduction of the binding ability and the loss of immunoreactivity.

Conclusion: this structural and functional approach turns out to be an effective way to interpret the conformational features that underline the binding capability of the target proteins and provide further information on the antigenic determinants of MUP.

References: 1) Ass. Occup. Hyg. (2002), 46(1), pp. 61-68. 2) J. Allergy Clin. Immunol. (2000), 106, pp. 1075-80. 3) Protein Science (2001), 10, pp.411-417.

Poster No. I-15

Isolation and Characterization of Betalactoglobulin from Reindeer Milk

Suutari T[1], Heikura J[1], Keskitalo H[1], Nieminen M[2] & Valkonen K[1], [1]Biotechnology Laboratory, University of Oulu, Sotkamo, Finland and [2]Reindeer Research Station, Finnish Game and Fisheries Research Institute, Kaamanen, Finland

Betalactoglobulin (BLG) is the main whey protein in most ruminants, and belongs to the lipocalin protein family. Properties of bovine BLG are well known while reindeer BLG has not been well characterized earlier. Our aim was to isolate and characterize reindeer BLG to investigate it's genetic variants, it's allerginicity to humans and it's role as a transport protein.

Reindeer milks were obtained from the Reindeer Research Station (Kaamanen, Finland). Milk fat was removed by centrifugation, and caseins and other whey proteins by isoelectric precipitations at pH 3.2. BLG remained in the supernatant and was further purified by gel filtration (Superdex-75) and by ion-exchange chromatography (Uno Q-1).

Our results show that the amino acid composition of reindeer milk BLG resembled that of bovine milk BLG. Reindeer milk BLG contains only three cysteines, while bovine BLG contains five cysteines. This may affect the three dimensional structure of reindeer milk BLG since cysteines play an important role in the formation of the three-dimensional structure. The molecular masses of the two proteins are similar while their isoelectric points differ indicating charge differences between the two proteins. Interesting is also that only one non-glycosylated genetic variant was detected in BLG purified from reindeer milk. However, further studies are needed to investigate if the structural differences detected in this study affect the possible biological transport function and allergic properties of reindeer BLG.

BENZON SYMPOSIUM No. 50

THE LIPOCALIN PROTEIN SUPERFAMILY

AUGUST 24-28, 2003, COPENHAGEN, DENMARK

Organizing committee:
Bo Åkerström (Lund), Darren Flower (Compton), Jean-Philippe Salier (Rouen) and Niels Borregaard (Copenhagen)

Abstracts - TUESDAY, August 26, 2003

Nitric Oxide Transport by Lipocalins From Blood-Sucking Insects
Montfort, WR, Dept. of Biochemistry & Molecular Biophysics, University of Arizona, Tucson, Arizona, USA
Recent gene sequence and crystal structure determinations of salivary proteins from several blood-sucking arthropods have revealed an unusual evolutionary relationship: many such proteins derive their functions from lipocalin protein folds. Many blood-sucking arthropods have independently evolved the ability to overcome a host organism's means of preventing blood loss (called hemostasis). Most blood-feeders have proteins that induce vasodilation, inhibit blood coagulation, and reduce inflammation, but do so by distinctly different mechanisms. Despite this diversity, in many cases the antihemostatic activities in such organisms reside in proteins with lipocalin folds.

Our lipocalin studies have centered on *Rhodnius Prolixus* – the kissing bug. This insect has at least twelve antihemostatic proteins in the saliva, ten of which are apparently in the lipocalin family. These proteins promote blood flow and reduce inflammation by delivering the vasodilator nitric oxide (NO), sequestering histamine, serotonin and ADP, preventing platelet aggregation, and inhibiting blood coagulation. *R. prolixus* is a South American bug that transmits, while feeding, the trypanosome responsible for Chagas' disease, which has no known cure and is endemic in South America.

The best characterized of these proteins are the nitrophorins, which store NO in the saliva via complexation with ferric heme. NO binding is tight (~ 10 nM) at the low pH of the saliva but is released on entering the higher pH of a victim's tissue. In the tissue, the nitrophorins bind tightly to histamine, again through heme, to reduce inflammation, and one nitrophorin is also an anticoaggulant. We have expressed and characterized four of the six *Rhodnius* nitrophorins, and determined crystal structures of three of these. I will present kinetic, crystallographic and computational results that support a model where diffusion of NO into and out of the binding pocket is controlled by mobile loops – which is somewhat counter- intuitive. Structures to 0.85 Å resolution will be presented in support of this model, and heme distortion by the protein will be suggested to stabilize the ferric heme required for activity.

Apolipoprotein D
Rassart E, Terrisse L. & Do Carmo, S, Département des sciences biologiques, Université du Québec à Montréal, Canada
Apolipoprotein D (apoD) is a 29 kDa glycoprotein that is primarily associated with high density lipoproteins in human plasma. Although apoD can bind cholesterol, progesterone, pregnenolone, bilirubin and arachidonic acid, it is unclear if any, or all of these, represent its physiological ligands. The apoD gene is expressed in many tissues, with high levels of expression in spleen, testes and brain. ApoD is present at high concentrations in the cyst fluid of women with gross cystic disease of the breast, a condition associated with increased risk of breast cancer. It also accumulates at sites of regenerating peripheral nerves and in the cerebrospinal fluid of patients with neurodegenerative conditions such as Alzheimer disease. ApoD mRNA and protein levels were also increased in the rat cortex after enthorinal lesioning. ApoD may, therefore, participate in maintenance and repair within the central and peripheral nervous systems. Furthermore, apoD mRNA is up-regulated following growth arrest in cell cultures.

Analysis of gene expression in cells transfected with constructs of the apoD promoter demonstrated that a pair of serum responsive elements (SRE) and a potential Z-DNA forming sequence are the major determinants of this growth arrest-induced apoD gene expression. While its role in metabolism has yet to be defined, apoD is likely to be a multi-ligand, multi-functional transporter. It could transport a ligand from one cell to another within an organ, scavenge a ligand within an organ for transport to the blood or could transport a ligand from the circulation to specific cells within a tissue.

Structure and Function of Histamine-binding Proteins and Related Lipocalins from Ticks
Paesen GC, CEH Oxford, Oxford, UK

Ticks are haematophagous arthropods that remain attached to the skin of their hosts for days to weeks. Successful feeding, therefore, requires suppression of inflammation at the feeding site. Histamine, a principal mediator of cutaneous inflammation, is sequestered by lipocalins secreted in the saliva of ticks. The brown ear tick, *Rhipicephalus appendiculatus*, secretes at least 3 different histamine-binding proteins (RaHBPs). Whereas lipocalins generally carry a single, hydrophobic ligand, the RaHBPs harbor two internal binding sites that appear designed to accommodate charged, hydrophilic ligands. In RaHBP2, one of the pockets (the *H* site) binds histamine with high affinity and is found at the position expected from other lipocalins. The second (*L*) site is a low-affinity site for histamine and is found at the end of the barrel that is closed off in other lipocalins. Typical lipocalin characteristics (such as the 3_{10} helix and a structural cluster of conserved residues) were apparently sacrificed to create the extra binding pocket. Salivary glands of other (Ixodid) tick species contain RaHBP-related lipocalins, most of which appear to have two distinct binding pockets. Some, but not all of these proteins bind histamine. Interestingly, a lipocalin isolated from *Dermacentor reticulatus* ticks binds histamine in its *H*-site, and serotonin (a mediator of inflammation in the rodent host of this species) in its *L*-pocket. Some of the tick lipocalins are monomers, whereas others are expressed as (covalently or non-covalently linked) homodimers. The degree of glycosylation also varies from one protein to the other. The tick lipocalins discussed all originate from the family of *Ixodidae* (hard ticks). They are distantly related to a family of presumably lipocalin-like proteins found in the other main tick family (Argasidae or soft ticks).

Structure and function of odorant binding proteins

Pelosi, P, Department of Agricultural Chemistry and Biotechnologies, Pisa, Italy

Odorant-binding proteins (OBPs) represent a sub-class in the lipocalin superfamily and are involved in the perception and transport of pheromones and odours in vertebrates.

They are secreted by glands present in the nasal area and are among the main protein components of nasal mucus. Other proteins, identical or very similar in their amino acid sequences to OBPs, are present in biological fluids, including urine and saliva, and are known under different names, such as MUPs (major urinary proteins) and SALs (salivary lipocalins). Unlike OBPs of the nasal area, they are associated with the specific pheromones when excreted, indicating their role in the delivering of chemical messengers in the environment. While nasal OBPs are present in both sexes, generally MUPs and SALs are sex specific and their synthesis is under hormonal control.

OBPs and related proteins share with all lipocalins the typical b-barrel structure. Sequence similarity, instead, is very poor and limited to few residues in addition to the short lipocalin signature –G-X-W-. Two conserved cysteines are present in most OBPs and connected by a disulphide bridge. However, some members of this family lack cysteines completely or present additional ones.

So far nearly all OBPs have been isolated from mammalian species. Each species expresses generally three sub-types, each represented by a small number of isoforms. Binding affinities to a number of potential odorants have been measured with several OBPs, indicating a very broad specificity with dissociation constants in the micromolar range.

Several characteristics of vertebrate OBPs seem to indicate that these proteins might be involved in the perception of specific pheromones, rather than general odorants.

Poster No. II-1

The *Drosophila* Lipocalin, *Karl*, is specifically expressed in the blood cell compartment

Bailey U-M, WGI Developmental Biology, University of Stockholm, Stockholm, Sweden

In *Drosophila*, studies of the blood cells has until a few years ago been focused on morphological properties. Only recently has studies at a molecular level been carried out. In a search for genes that were specifically expressed in the blood cells, we found a putative lipocalin. We made a tissue specific cDNA microarray chip based on random clones from a *Drosophila* blood cell cDNA library, that we had generated. RNA were isolated and labeled from five different mutants that either over-proliferate or have fewer circulating blood cells. RNA from one blood cell line were also labeled and compared to standard wild type RNA. About 150 clones were picked for sequencing and further computer analysis. As a secondary screen *in situ* hybridizations with 20 genes were carried out. Several genes showed a blood cell specific expression pattern. One gene, in particularly, had an interesting expression pattern. We have named this gene *Karl*. *Karl* share sequence similarities to lipocalins. Microarray experiments and Northern blot analysis demonstrate that *Karl* is highly expressed in the *Drosophila* blood cell line mbn-2. *In situ* hybridizations using the *Karl* cDNA resulted in a strong signal in the circulating blood cells and in the lymph glands, which is the larval hematopoetic organ. We have made transgenic flies carrying putative regulatory elements of the *Karl* gene fused to the GAL4 gene and we are currently analyzing the offspring. Blood cells are playing an important role in the immune response as macrophages and producers of antimicrobial peptides. Currently we are investigating if *Karl* is involved in this processes.

Poster No. II-2

Genetic Analysis of the Lipocalin Lazarillo Function: A Multiorganismal Approach

Ganfornina MD[1], Sánchez D[1], Lora JM[2], Torres-Schumann S[3], Voguel M[3], Martínez S[4] & Bastiani MJ[3]. [1]IBGM, U. of Valladolid, Spain, [2]Millennium Pharmaceuticals, Boston, USA, [3]Biology Dept. U. of Utah, USA, [4]Instituto de Neurociencia, Alicante, Spain

We are interested in understanding the function of Lazarillo (Laz), a lipocalin found to have a role in axon guidance in the grasshopper embryo. Because of this unique function in the family, we expanded our approach to Laz homologous proteins in genetically manipulable model organisms. Phylogenetic analyses of the family show that Apolipoprotein D (ApoD), a well known lipocalin, is orthologous to Laz. We found two Drosophila genes, Nlaz and Glaz, to be the closest relatives to grasshopper Laz.

All Laz genes are expressed in subsets of cells in the nervous system (NS) during embryogenesis. Grasshopper Laz and Drosophila Nlaz are restricted to subsets of neurons, while Drosophila Glaz is glial specific. Mouse ApoD is expressed by neural crest-derived embryonic mesenchymal cells that give rise to meninges, and by pericytes surrounding NS capillaries. However, a subset of neurons and glia start expressing ApoD during postnatal NS development.

We have generated null mutant alleles for Nlaz, Glaz and ApoD. Deletions encompassing the 5'end and the first exon of Glaz were generated by imprecise excision of a nearby P element. A point mutation introducing a stop codon in the third exon of Nlaz was introduced using targeted mutagenesis by homologous recombination. The ApoD KO mouse was generated by an insertional mutation in the 6th exon. Preliminary results show that all the null mutants are viable, suggesting that loss of one lipocalin produces only subtle effects under normal laboratory conditions. ApoD KO mice show a decreased performance in maze tests and a lower number of Purkinje neurons. To further analyze the loss-of-function mutants we are taking two directions: 1) To test functional redundancy, by generating double mutants, and 2) To test the mutations on NS development under special stress conditions.

Poster No. II-3

Uterocalin/24p3, an acute phase protein expressed by the mammary gland

Zhao W, Ryon J, Bendickson L & Nilsen-Hamilton, M, Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA, 50011, USA

The lipocalin, uterocalin (SIP24/24p3), is an acute phase protein. Characteristic of this and other acute phase proteins is its production by the liver and other tissues in response to inflammation or toxic challenge. Uterocalin is also produced in large quantities during involution of mammary gland and uterus. At its peak, the level of uterocalin protein expression in these tissues reaches an average of 0.2-0.5% of the total extractable protein. Uterocalin expression in the mammary gland is also regulated by the estrous cycle.

The period of uterocalin expression during involution is consistent with the hypothesis that one of its physiological roles is to induce apoptosis of invading neutrophils, as shown by others, and to delay the entry of neutrophils into the tissue until the second phase of involution. Recent results show that uterocalin expression remains higher in primiparous gland than in virgin glands after the pregnant glands have completely involuted. This observation and the known protective effect of early pregnancy on later development of breast cancer suggests that the ability of uterocalin to induce apoptosis in neutrophils might also decrease oxidative and carcinogenic activity in the gland and result in a lower mutation rate and thus a lower probability of cancer in the primiparous gland.

Poster No. II-4

The lipocalin α_1-microglobulin generates superoxide radicals

Allhorn M[1], Klapyta A[1,2], Park J[3], Osmark P[1], Nilson BHK[4] & Åkerström B[1], [1]Department of Cell and Molecular Biology, Lund University, Lund, Sweden, [2]Department of Biochemistry and Molecular Biology, Jagiellonian University, Krakow, Poland, [3]Department of Biochemistry, Lund University, Lund, Sweden and [4]Active Biotech AB, Lund, Sweden

α_1m is a 26 kDa extracellular plasma and tissue glycoprotein found in most or all vertebrates. It belongs to the Lipocalin protein superfamily, a group of proteins in bacteria, plants and animals with a conserved three-dimensional structure but widely diverse functions. The lipocalin fold consists of a β-barrel forming a pocket with a hydrophobic interior. α_1m has a heterogeneous yellow-brown chromophore consisting of small unidentified prosthetic groups localized to a free thiol group (C34) and three lysyl residues (K92, 118 and 130) around the entrance to the lipocalin pocket. It was recently reported that α_1m can bind heme and that a C-terminally processed form of α_1m degrades heme concomitantly with formation of the yellow-brown chromophore. The mechanisms of the heme-binding and degradation-reactions are unknown.

It is shown here by three different methods that plasma and recombinant human α_1m can generate superoxide radicals. First, the proteins reduced the heme-protein cytochrome c and the reduction was inhibited by superoxide dismutase (SOD). The reduction of cytochrome c was enhanced by the addition of NAD(P)H, suggesting an NAD(P)H oxidase-like activity of α_1m. Second, the chromogenic compound nitroblue tetrazolium (NBT) was reduced by α_1m and this reaction was also inhibited by SOD. Third, lactate dehydrogenase catalysed NADH-oxidation in the presence

of a1m, under conditions which require superoxide radicals. Recombinant mutated a_1m-forms lacking the chromophore-carrying C34, K92, K118 and K130 residues displayed a weaker cytochrome c-reduction activity, suggesting that the chromophore is involved in the reaction. The superoxide generation may be important for the heme-binding and degradation mechanisms of α_1m.

β-Lactoglobulin – Structure, Properties and Function

Sawyer L[1], Kontopidis G[1], Wu S[1], Holt C[2], Jayat D[3] & Haertlé T[3], [1]ICMB, The University of Edinburgh, Edinburgh EH9 3JR, [2]The Hannah Research Institute, Ayr, KA6 5HL, Scotland, [3]LEIMA-INRA, BP 71627, 44316 Nantes Cdx 03, France

β-Lactoglobulin (β-Lg) is the major whey protein in ruminant milk and it is found in many, but not all, milks. The protein is a core member of the lipocalin family and as such binds small, generally hydrophobic ligands like palmitate and retinol, but as the protein is abundant and easy to prepare, a large number of other ligand binding studies have been reported as well as a wide variety of physicochemical studies using effectively every known technique. The crystal structures of the protein from several species have been reported and provide significant insight into the dimeric nature of the bovine protein. Several mutations that affect this have been carried out by us and by others. Further, the reactivity of the free thiol, Cys121, leads to dimer dissociation and the mutation Cys121Ser will be discussed in the light of this. Some of the properties of β-Lg that may give clues as to the physiological function of β-Lg will also be discussed with the proposed putative function of the protein in mind. However, by consideration of the species distribution of β-Lg together with these other studies, the true physiological function of the protein will be proposed: the presence in milk of β-Lg is largely for nutritional purposes while the original function is in some aspect of foetal development at an early stage in gestation – β-Lg in milk is a convenient nutritional protein derived from glycodelin.

Siderocalin, Siderophores and Iron

Strong RK, Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Siderocalin (also known as neutrophil gelatinase associated lipocalin (NGAL), 24p3, uterocalin and *neu*-related lipocalin) is found in neutrophil granules, uterine secretions and secreted from epithelial cells in response to inflammation or tumorigenesis. Siderocalin is also an acute phase protein, with markedly elevated levels in the serum and synovium during bacterial infection. By sequence homology, siderocalin is a member of the lipocalin protein family: secreted proteins that generally bind to and transport hydrophobic, small-molecule ligands. Though implicated in diverse physiological processes, the precise role of siderocalin was mysterious until our recent identification of a high-affinity (K_D = 0.4 nanomolar) ligand for this protein: the catecholate-type bacterial ferric siderophore enterochelin (or enterobactin). Unlike typical lipocalin ligands, ferric enterochelin is charged and interacts with siderocalin using a novel recognition mechanism that incorporates the potential for considerable cross-reactivity. Indeed, siderocalin has now been shown to bind a variety of distinct bacterial siderophores. We have proposed that siderocalin functions as a bateriostatic agent, sequestering iron as ferric siderophore complexes, thus complementing the multi-pronged anti-microbial iron-depletion strategy of the mammalian innate immune system. Siderocalin is a potent bacteriostatic agent *in vitro* under iron-limiting conditions. The siderocalin functional hypothesis also explains the association of some siderophores with virulence: by alternately utilizing siderophores that show markedly reduced affinity for siderocalin, pathogens can escape siderocalin-mediated iron-deprivation. Siderocalin has also been shown to act in a transferrin-independent iron delivery pathway, operational in specific mammalian cell types, through an association with an, as yet, unidentified mammalian 'siderophore'.

Poster No. II-6

Stimulation of Male Behaviour by Urinary Protein and Estrus Specific Volatiles in Female Mice (*Mus musculus*)

Achiraman, S & Archunan G, Department of Animal Science, Bharathidasan University, Tiruchirappalli-620 024, Tamilnadu, India

Soluble proteins of low molecular mass seem to play an important role in chemical communication. They are present at high concentrations in biological fluids and involved in delivery of chemical messages of pheromonal significance. Both mice and rat secrete pheromones in urine and deposit these signals as scent marks throughout their home ranges. Two different sex pheromones exists in the female mouse urine, (i) a potent, but ephemeral pheromone whose activity disappears in less than 24 hours and (ii) a stable, but less potent pheromone, whose activity can be detected for at least 30 days. Certain specific compounds namely isocroctylamine, 4-methyl-2-heptanone and azulene of proestrus stage and the compounds, 1-H-cyclopop.e.azulene, caryophyllene, copanene of estrus stage have been identified in mice (*Mus musculus*) (Achiraman, 2002). Major Urinary Protein (MUP) has been reported to act as carrier of chemical signals in mice. The present investigation was design to evaluate the role of urinary protein and its involvement along with identified urinary compounds in pheromonal communication by studying body rubbing and grooming behaviour in male mice. The results indicated that the estrus specific compounds 1-iodo-2-methylundecane, azulene significantly enhanced the grooming behaviours in males. The proestrus specific compound 4-methyl 2-heptanone is involved in

both the behavioural activities. Body rubbing was found to be more towards the castrated mice smeared with the urinary protein and it is comparable to that of male behaviour towards estrus females. Both the identified compounds with urinary protein exhibited high level of body rubbing and grooming behaviour than when they were exposed individually. The present study provides evidence that mixture of compounds is probably involved in pheromonal communication rather than a single compound, even though the single compound has got its unique function in behavioural activities. Hence, this study concludes that urinary protein may also act as pheromone in spite of being a odourant carrier. Further, the use of this information will aid us in developing a new technology (Pheromonal trap) to control the rats using non-lethal pest management.

Poster No. II-7

A protective role of OBP against lipid peroxidation damage is suggested by its ability to bind to 4-hydroxy-2-nonenal (HNE) and resist to its chemical activity

Ramoni R, Merli E, Conti V & Grolli S, Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Università di Parma, Italy

Mammalian nasal mucosa is constantly exposed to the injuries of reactive oxygen species (ROS) generated by oxygen and other airborne compounds flowing throughout nasal cavities. In this work we investigated the putative protective role of Odorant Binding Protein (OBP) against the activity of ROS in the hypothesis that OBP may behave as a scavenger for aldehydes derived from lipid peroxidation. To prove this role, we tested the binding properties of bovine and porcine OBPs with respect to HNE. This reactive aldehyde, whose tissue levels are reported to be usually μM (1 – 5) and transiently mM (up to 5), can form stable covalent adducts with proteins and nucleic acids leading to their inactivation. The experimental results of competitive assays with the fluorescent ligand 1-amino-anthracene (AMA) indicated that the binding to HNE was completely reversible and that the Kd values (5.1 and 3.2 μM for porcine and bovine OBP, respectively) were comparable to those of other "good ligands" of OBPs. In addition, we realized direct AMA binding tests with OBPs pre-incubated in the presence of HNE, at concentration 0.1-2.5 mM. The results showed that significative chemical modification of OBP was detected (western blotting with an antiserum reacting with HNE-protein Michael adducts) starting from 0.25 mM HNE, but the binding capacity for AMA was partially lost for HNE levels higher than 1.0 mM. Binding properties and chemical resistance to HNE, suggest that OBP, when present in sub-mM concentrations, might be a plausible scavenger for this aldehyde at the levels found in most tissues in vivo.

Poster No. II-8

Role of Urinary Proteins In Bovine: A New Light In Chemical Communication

Kumar, K Ramesh & Archunan, G, Department of Animal science, Bharathidasan University, Tiruchirappalli-620 024, Tamilnadu, India

Successful reproduction in many species is facilitated by chemical communication. The female produces a specific odour during estrus through urine by which male exhibits a variety of behaviour following the perception of estrus odour before mating. Urinary proteins play a major role as pheromones in mammalian reproduction and social behaviour. The persistence of the pheromone activity is less than 24 hours and it is less potent pheromone, whose activity can be detected for long period. The estrus-specific compound 1-iodoundecane has been identified in bovine estrus urine (Ramesh Kumar et al., 2000). Earlier reports indicated that urinary proteins can act as a carrier molecule for the chemical signals. So, the present investigation was planned to estimate the urinary proteins and their role in pheromonal communication. The protein content found to be varied in the bovine urine of all reproductive phases, which may be due to the changes in hormonal profile. However, another important finding of the present study is that the proteins are highly excreted through urine during estrus phases. These urinary proteins (non-volatile) would stimulate the male to generate the appropriate mating behaviour towards female. Hence the urinary protein may have a relation with sexual behaviour in bovine. Further investigation is needed to confirm this hypothesis.

Poster No. II-9

Glycodelin A: a stress inducing protein which triggers apoptosis in T cells

Mukhopadhyay D & Karande AA, Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India.

Glycodelin is a secretory lipocalin expressed mainly by primate reproductive tissues. The protein is 54.6 % identical to equine beta-lactoglobulin (bLG), but unlike bLG, glycodelin has complex N-linked glycosylation which is important for its contraceptive function. Till date no ligand for this lipocalin has been reported. Glycodelin is a multifunctional protein having contraceptive, immunosuppressive, angiogenic and morphogenic properties. We have been interested in understanding the molecular mechanism involved in the immunosuppressive function of glycodelin. Recently we have demonstrated that glycodelin A (GdA; the isoform present in amniotic fluid) induces apoptosis in activated T cells independent of macrophage participation.

Programmed cell death can be initiated either at the plasma membrane by the receptors like Fas (CD96), TNFR and DR, or it can be initiated by signaling to the mitochondria which leads to opening of the mitochondrial permeability transition pore (PTP) and spillage of several pro-apoptotic molecules into the cytoplasm. Our studies suggest that

induction of apoptosis by GdA is caspase dependant but does not engage Fas in Jurkat cells. Further studies using a caspase 8 knock-out strain of Jurkat cells (A3 I9.2) have shown that GdA-induced apoptosis is independent of initiator caspase 8 and can be partially rescued by the caspase 9 inhibitor. Activation of JNK, the pro-apoptotic MAP kinase, is also not involved in GdA induced cell death. After addition of GdA to cells, the mitochondrial membrane potential was lost within 4-6 h. Hence it is clear that GdA triggers death signals by damaging the mitochondria, however the mechanism is yet to be elucidated. All these evidence indicate that GdA can induce stress on T cells which is sensed by the mitochondria leading to apoptosis.

Poster No. II-10

Expression of retinol-binding protein in the liver and ovary during oocyte development in the Rainbow Trout

Lissauer L, Sammar M, Levavi-Sivan B, Avarre J-C & Lubzens E, Israel Oceanographic and Limnological Research, Haifa, Israel

In the chicken, retinol is the main retinoid in eggs and is delivered to the developing oocytes by retinol-binding protein (RBP). While vitellogenin (VTG) is suspected as the main carrier of carotenoids and retinals to fish eggs, the origin of retinols and retinyl-esters in fish oocytes has yet to be resolved.

Studies were initiated to determine whether plasmatic retinol-binding protein contributes retinols to developing fish oocytes. Results show that mRNA levels in the liver and RBP plasma levels did not change significantly with the onset and during vitellogenesis in Rainbow trout. This is in contrast to hepatically synthesized proteins (such as VTG) that are incorporated into the developing oocyte. A dramatic elevation in the mRNA levels of VTG and an increase in VTG plasma levels were found in the same females, sampled at various stages of vitellogenesis. These results for Rainbow trout, are similar to those reported for the chicken, but differ from those of *Xenopus*, where treatment with 17b-estradiol resulted in an increase in RBP mRNA in the liver and of retinal and retinol levels in the plasma.

As RBP was localized in the cytosol of ovulated fish oocytes, we investigated whether it originates from the plasma as reported for the chicken oocyte or from oocytes, granulose or theca cells. In trout, RBP transcripts were found by RT-PCR in the ovary, oviduct (the ovarian tissue adjacent to the gonopore) and in ovulating oocytes, suggesting a modulating role for RBP in follicular development. The relative abundance of the RBP transcripts in the ovary, oviduct and oocytes, during vitellogeneis, is currently under investigation, using real-time PCR. These results indicate that while the most systematic studied model is the chicken egg, it may not be suitable to other oviparous species.

Poster No. II-11

Binding of α1-microglobulin to heme immobilized on agarose beads: a tentatively useful tool for the isolation of α1-microglobulin homologues in new species

Larsson J, Allhorn M & Åkerström B, Department of Cell and Molecular Biology, Lund University, Lund, Sweden

The aim of this study was to further investigate the recently discovered binding of heme to α1-microglobulin, using heme immobilized on agarose beads.

Background: The lipocalin α1-microglobulin (a1m), a.k.a. protein HC, found in plasma and tissues, has a brown colour, forms covalent complexes with other proteins and has immunomodulatory effects in vitro, but the physiological function is not yet established. It has been found in mammals, amphibians and teleost fish indicating conservation during evolution. Human a1m was recently shown to bind heme and, after cleavage of a C-terminal tetrapeptide induced by erythrocyte membranes or purified hemoglobin, initiate heme degradation. It has thus been assigned a putative role as a heme scavenger.

Methods: Heme immobilized on agarose beads was incubated with whole plasma, purified free and complexbound human a1m, sera from several species and different bodily fluids, respectively. Bound material was eluted and analysed using SDS-PAGE, Western blotting and RIA.

Results: A1m displayed a concentration dependent binding to heme-agarose compatible with specific binding. A1m, identified on Western blotting after SDS-PAGE, was found in eluates from heme-agarose after incubation with sera from nonhuman species, as well as in human bodily fluids.

Conclusions: Heme-agarose binds a1m both in plasma and in purified form. The ability to bind heme, previously reported for human a1m, is shared with a1m-homologues from several other species indicating that this property of a1m has been conserved during evolution and suggesting a functional importance. Binding to immobilized heme could be utilized in attempts to isolate new a1m-homologues.

Poster No. II-12

Complete refolding of bovine β-lactoglobulin requires disulfide bond formation by the strictly regulated reducing method

Hattori M[1], Hiramatsu K[1], Kurata T[1], Nishiura M[1], Takahashi K[1], Ametani A[2] & Kaminogawa S[2], [1]Tokyo University of Agriculture and Technology, [2]The University of Tokyo, Tokyo, Japan

When β-lactoglobulin (β-LG) was denatured with 6 M guanidine hydrochloride (GdnHCl) containing 2-mercaptoethanol and subsequently dialysed against phosphate-buffered saline (PBS), refolding of this protein was

incomplete in spite that a biological activity of retinol-binding was almost recovered similarly to that of the native molecule (Hattori *et al.*, *J. Biol. Chem*. **268**, 22414-22419 (1993)). Exposure of hydrophobic region(s) and incorrect disulfide bond formation were found for such dialyzed β-LG molecules, shown by the enzyme probe method, evaluation of hydrophilicity values, in-gel mobility on SDS-PAGE and evaluation of disulfide bonds with the Ellman method. It was revealed that complete refolding was attained by dilution of denatured β-LG with PBS containing the reducing agent, and slow reoxidation of sulfhydryl groups on dialysis for gradient removal of the reducing agent with 6 steps. Complete renaturation was confirmed by analyzing retinol-binding activity, CD spectra, intrinsic fluorescence measurement), binding ability of monoclonal antibodies and SDS-PAGE. Step-by-step disulfide bond formation was considered to be critical for complete refolding of denatured β-LG. Our method can contribute to establish the method for complete refolding of useful recombinant proteins *in vitro* without biological aids such as chaperones.

Poster No. II-13

Expression and characterization of a truncated form of bovine Odorant Binding Protein consisting of the beta-barrel domain

Grolli S[1], Spinelli S[2], Conti V[1], Merli E[1], Tegoni M[2], Cambillau C[2] & Ramoni R[1], [1]Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti, Università di Parma, Parma, Italy, [2] Architecture et Fonction des Macromolecules Biologiques, UMR 6098 CNRS, Marseille, France

In order to elucidate the role of the alpha-helix on folding and ligand binding properties of bovine Odorant Binding Protein (bOBP), we have introduced a stop codon in the cDNA of the native isoform at the position corresponding to Asn 122, and produced a truncated form (residues 1-121). This mutant (mini bOBP), lacking the hinge and the alpha-helix sequences, is formed only of the beta-barrel, i.e. the domain that contains the ligand binding site. Ion exchange chromatography on Resource Q column resolves mini-OBP in two different non-interchangeable peaks, probably corresponding to alternative conformational states. Both forms (mini bOBP I and II) have the expected molecular mass, are correctly folded (CD) and show a binding capacity for the fluorescent ligand 1-amino-anthracene comparable to that of native bOBP. Dynamic light scattering experiments indicate that, in the presence of 250 mM NaCl, both forms behave as monomers, while mini bOBP I partly aggregates in the absence of salt. Preliminary trials have led to the crystallization of the mini bOBP form I. Further structural and functional studies will be pursued to understand the peculiar characteristics of the two mini bOBP forms.

Poster No. II-14

Characterisation of the Unfolding Intermediate States of Porcine Beta Lactoglobulin

D'Alfonso L[1], Collini M[1], Molinari H[2], Ragona L[3], Catalano M[2] & Baldini G[1], [1]Università degli Studi di Milano-Bicocca, Dipartimento di Fisica, [2] Università degli Studi di Verona, Dipartimento Scientifico e Tecnologico, [3] ISMAC, CNR, Via Ampere 56, 20131 Milano, Italy

In the present study the denaturation of porcine beta-lactoglobulin (PLG), induced by guanidinium hydrochloride (GuHCl), has been studied in order to establish its chemical stability compared to that of the well known bovine analogue (BLG). Bovine and porcine beta-lactoglobulins belong to the lipocalin family, share the same beta-barrel fold but, in spite of their high sequence similarity (63% identity, 80% similarity) they exhibit a very different monomer-dimer equilibrium versus pH: PLG is monomeric at neutral pH, while BLG is monomeric at acidic pH. This behaviour points to the important role played by the charged residues on the stability and physico-chemical properties of these proteins. In particular PLG in the acid dimeric state has been found to be less stable than in its monomeric neutral state, in analogy with the behaviour observed for BLG, which is less stable in its dimeric state, at neutral pH, thus indicating that both proteins are more stable as monomers.

The denaturation profiles of PLG, as obtained at two different pH values, 6 and 2, by i) tryptophan fluorescence; ii) near and far UV circular dichroism and iii) ^{1}H NMR spectroscopy show the existence of at least two intermediates. The lowest GuHCl concentration (<0.1M) intermediate state can be ascribed to a conformational change induced by ionic strength, in analogy with the results obtained for BLG. The other intermediate state occurs at a higher GuHCl concentration (1-1.5 M) and the fitting to a multi-state denaturation model allows the determination of the thermodynamic parameters.

BENZON SYMPOSIUM No. 50

THE LIPOCALIN PROTEIN SUPERFAMILY

AUGUST 24-28, 2003, COPENHAGEN, DENMARK

Organizing committee:
Bo Åkerström (Lund), Darren Flower (Compton), Jean-Philippe Salier (Rouen) and Niels Borregaard (Copenhagen)

Abstracts - WEDNESDAY, August 27, 2003

Crystal Structures of Pheromones and Odor Transport Lipocalins
Cambillau CH, Architecture et Fonction des Macromolécules Biologiques, CNRS-UMR 6098, Marseilles, France
In mammals, the olfactory system is able to recognise a wide range of different odorants. The perception of particular odorants may produce cultural or instinctive behaviour, the latter probably depending on an unconscious recognition process which subsequently initiates defined social or sexual responses. The *olfactory epithelium* and the *vomeronasal organ* [4] are two functionally and anatomically distinct tissues devoted to these two types of perception. The molecules invoking response by the vomeronasal system are traditionally called pheromones. The carrier proteins, OBPs and PBPs have been shown to belong to the lipocalin family.

I will report on the structures of bovine OBP (OBPb), porcine OBP (OBPp), hamster aphrodisin and Boar Salivary Lipocalin (SAL). OBPb, a homodimer of 2 x159 amino acids, is secreted in millimolar concentration by the nasal respiratory epithelium. The protein has the typical fold of a lipocalin but with one startling difference: the single helix of one monomer interacts with the ß barrel of the other, thus displaying the so-called 'domain-swapping', likely associated with the absence of any disulfide bridge. By combining mass spectrometry, X-ray crystallography (1.8 Å resolution) and fluorescence, it has been unambiguously established that natural OBPb contains the racemic form of 1-octen-3-ol, which is a typical component of bovine breath. 1-octen-3-ol is also an extremely potent olfactory attractant for many parasite vectors like *Anopheles* (*Plasmodium*) or *Glossina* (*Trypanosoma*). We have solved the structure of OBPb and OBPp with various odorant molecules and assayed their binding with fluorescence. Aphrodisin, a hamster PBP, produced and secreted in the vaginal discharge of the *female* induces sexual stimulation of the male. The structure of recombinant aphrodisin, solved at 1.6 Å resolution, revealed the presence of a disulfide bridge characteristic of a lipocalin-subfamily mainly found in rodents, and is analogous to the 5th disulfide bridge of the long toxins, such as alpha cobratoxin. We have also solved the structure of SAL, a pheromone-binding protein specifically expressed in the submaxillary glands of the boar, at 2.1 Å resolution.

Retinol Binding Protein: Structure and Function
Newcomer M, Louisiana State University, Baton Rouge, LA, USA
A homeostatic level of retinol in plasma serves as a constant source of the precursor for the active vitamin-A-derived retinoids all-*trans* and 9-*cis* retinoic acid (regulators of gene transcription) and 11-*cis*-retinal (the chromophore of rhodopsin). In plasma vitamin A in the form of all-*trans*-retinol is bound to the lipocalin Retinol Binding Protein (RBP) and it is this protein that mediates the transport and delivery of the vitamin to the target tissues. RBP is synthesized primarily in the liver, where it requires the binding of retinol to trigger its secretion. RBP is found complexed with transthyretin (TTR) in plasma and it is this protein:protein interaction which prevents excessive loss of RBP through glomerular filtration. X-ray structures have been determined for the holo and apo forms of RBP, as well as RBP in complex with TTR. Conformational changes associated with the different intermolecular interactions in which RBP participates have been described: retinol binding induces a localized conformational change in the ligand binding cavity, and transthyretin binding involves the ordering of a C-terminal extension found in mammalian RBP's. The numerous structures available for this lipocalin provide insights into understanding the function of the protein and the biological consequences of naturally occurring mutations or altered forms of RBP.

Conformation and folding of bovine β-lactoglobulin
Goto, Y, Institute for Protein Research, Osaka University , Osaka, Japan
The α-helix to β-sheet transition of proteins is a key issue for understanding the folding and biological function of a number of proteins. Bovine β-lactoglobulin (β-lg) will be a useful model for clarifying the mechanism of the α→β transition, since its folding process is accompanied by the α→β transition due to the inconsistency of local and non-local interactions. To define the structural and dynamic properties of an early folding intermediate in β-lg, the kinetics of folding was measured over the 100 ms to 10 s time range, using ultra-rapid mixing techniques in conjunction with

fluorescence detection and hydrogen exchange labeling probed by heteronuclear NMR [1]. The results indicate that efficient folding, despite some local non-native structural preferences, is insured by the rapid formation of a native-like α/β core domain.

We also studied the mechanism of the monomer-dimer equilibrium of β-lg [2]. Bovine β-lg exists as a dimer at neutral pH, while it dissociates to a native monomer below pH 3. Several site-directed mutants in which intermolecular interactions stabilizing the dimer would be removed were expressed and their monomer/dimer equilibria were studied by analytical ultracentrifugation. These results suggested that protein-protein interactions of bovine β-lg can be manipulated by redesigning the residues on the interface without affecting global folding.

[1] Kuwata *et al.*(2001) *Nature Struct. Biol.* **8** (2), 151-155.
[2] Sakurai and Goto (2002) *J. Biol. Chem.* **277** (28), 25735-25740.

Folding and interaction studies of -lactoglobulins and liver basic fatty acid binding protein

Molinari H, Università degli Studi di Verona, Verona, Italy

Our group has been involved in the last few years in the NMR structural study of proteins belonging to the lipocalin and fatty acid binding protein (FABP) families. Our NMR structural study is aimed at elucidating, through the comparative analysis of members of these two families, subtle changes brought about by evolution within the same superfamily to unravel the main determinants of aggregation properties, folding and ligand binding. The properties of bovine and porcine β-lactoglobulins (BLG, PLG) and chicken liver basic FABP (Lb-FABP) will be discussed in this presentation.

BLG and PLG aggregation properties: Dimerisation properties of the two proteins will be compared. Indeed PLG, showing 62% identity and 83% similarity with BLG, was shown, on the basis of NMR data and size-exclusion chromatography, to exhibit a monomer-dimer equilibrium with a pH dependence opposite to that found for BLG. The analysis of the electrostatic properties at the surface of BLG and PLG indicated that the two proteins could not share the same dimer interface.

BLG and PLG folding properties: Equilibrium unfolding and hydrogen/exchange NMR studies have been performed on BLG and the results compared to those obtained for peptides covering different BLG regions. PLG preliminary folding studies revealed a lower stability with respect to BLG.

BLG and PLG interaction studies: The binding properties of the two proteins will be discussed in term of the role of the Tanford transition. Docking experiments coupled to fluorescence competition studies will be discussed

Chicken Lb-FABP. Lb-FABP belongs to the basic-type fatty acid binding proteins and binds fatty acids and bile acids. The characterisation of folding and binding properties of this protein is in progress, with the aim to compare the data with those obtained for β-lactoglobulins.

The preparation of specific mutants is in progress for all the proteins, with the aim of completing the comparative analysis.

Poster No. III-1

Functional and Structural Analyses of Lipocalin-type Prostaglandin D Synthase (Beta-Trace)

Urade Y, Eguchi N, Irikura D, Ago H, Miyano M & Hayaishi O, Osaka Bioscience Institute, Osaka, Japan

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) catalyzes the isomerization of PGH_2, a common precursor of various prostanoids, to produce PGD_2, a potent endogenous somnogen and an allergic mediator. L-PGDS is localized in the central nervous system and male genitals of various mammals and also in the human heart. L-PGDS gene-knockout mice are devoid of touch-evoked pain induced by PGE_2 and exhibit poor rebound non-rapid eye movement (NREM) sleep after sleep deprivation. Human L-PGDS-overexpressing transgenic mice exhibit excess amounts of NREM sleep after noxious stimulation, with a concomitant increase in PGD_2 content in their brain. Therefore, L-PGDS is considered to contribute to the regulation of nociception and NREM sleep by producing PGD_2 in the central nervous system. Moreover, L-PGDS is secreted into various body fluids and is identical to beta-trace, which is a major component of human cerebrospinal fluid. L-PGDS binds biliverdin and bilirubin with high affinities (K_d = 30 to 40 nM). Increased production of L-PGDS was found in the cerebrospinal fluid of patients with subarachnoid hemorrhage. Thus, L-PGDS may bind those harmful heme degradation products produced after brain hemorrhage to eliminate them from the brain. L-PGDS is upregulated in oligodendrocytes of genetic demyelinating "twitcher" mice to protect against apoptosis of oligodendrocytes and neurons, and in the brains of patients with multiple sclerosis and several other neurodegenerative diseases, such as Tay-Sachs and Sandhoff diseases. We recently determined the crystal structure of recombinant mouse L-PGDS and elucidated the catalytic mechanism by site-directed mutagenesis based on the crystal structure.

Poster No. III-2

Nature of Sequence and Structural Conservation in the Lipocalin Superfamily: Relationship to Folding and Stability

Greene L*,† & Brew K*, University of Miami School of Medicine, Department of Biochemistry and Molecular Biology, Miami, Florida, U.S.A. and †Oxford Centre for Molecular Sciences and Department of Chemistry, Central Chemistry Laboratory, University of Oxford, UK

We will present the nature of sequence and structural conservation within the Lipocalin superfamily (1) derived from exhaustive and detailed computational and statistical approaches (2-4). The results of experimental studies conducted to explore the role of conserved residues in folding (4) and stability (5) using a model lipocalin, human serum retinol-binding protein, will be discussed. In summary we identify five evolutionarily conserved regions that form a mixed-polar/nonpolar core that closes the base of the barrel and comprises a significant proportion of the critical long-range interaction network within the b-barrel topology. Our experimental and theoretical results suggest that the conserved residues within these regions are key to the stability of the lipocalin fold and further we propose that they form an inherently stable and conserved substructure (termed the fold-determining core) during the early stages of the folding process that directs correct folding in relevant biological time.

References: (1) S. Pervaiz and K. Brew (1985) Science 228, 335-337. (2) L. Greene and K. Brew (1995) Protein Eng. 8, supplement, 100. (3) K. Brew and L.H. Greene (1997) Protein Eng. 10, supplement, 44. (4) L. H. Greene et al. (manuscript submitted) *Conserved signature proposed for folding in the lipocalin superfamily*. (5) L. H. Greene et al. (2001) Protein Sci. 10, 2301-2316.

Notes: This presentation is dedicated in memory of Syed Pervaiz.

Poster No. III-3

The molecular basis of the coloration mechanism in lobster shell: beta-crustacyanin at 3.2 Å resolution

Cianci M*, Rizkallah PJ†, Olczak A*, Raftery J*, Chayen NE‡, Zagalsky PF§ & Helliwell JR*, *Department of Chemistry, University of Manchester, Manchester, UK; †CCLRC, Daresbury Laboratory, Daresbury, UK; ‡Biological Structure and Function Section, Division of Biomedical Sciences, Faculty of Medicine, Imperial College, London, UK; §Department of Molecular Biology and Biochemistry, Royal Holloway College, University of London, Egham, Surrey, UK. Current Address: Institute of General and Ecological Chemistry, Technical University of Łodz, Łodz, Poland

Database deposition: The final coordinates and structure factors have been deposited with the Protein Data Bank (code 1GKA).

The binding of the carotenoid astaxanthin in the protein multi-macromolecular complex crustacyanin is responsible for the blue coloration of lobster shell. The structural basis of the bathochromic shift mechanism has long been elusive. A change in the colour occurs from the orange red of the unbound astaxanthin (λmax 472 nm in hexane), the well-known colour of cooked lobster, to slate-blue in the protein bound live lobster state (λmax 632 nm in crustacyanin). Intriguingly, extracted crustacyanin goes red upon dehydration and on rehydration goes back to blue. Recently the innovative use of softer X-rays and xenon derivatisation yielded the 3-D structure of the A1 apoprotein subunit of crustacyanin. This has now provided the molecular replacement search model for a completely new crystal form of the beta-crustacyanin holo complex, that is an A1 with A3 subunit assembly including two bound astaxanthin molecules. We have thereby determined the structure of the A3 molecule de novo, and also the structural chemistry of the biological coloration mechanism at the detailed molecular level in this beta-complex. Lobster has clearly evolved an intricate structural mechanism for the coloration of its shell utilizing astaxanthin and a bathochromic shift. Blue/purple caroteno-proteins are ubiquitous amongst invertebrate marine animals, particularly the Crustacea. For the first time, 3-D structural results on such a coloration mechanism are now available with our study on the lobster shell.

1. N E Chayen, M Cianci, A Olczak, J Raftery, P J Rizkallah, P F Zagalsky and J R Helliwell, (2000) Acta Crystallographica Section D-Biological Crystallography D56, 1064-1066.
2. M Cianci, P J Rizkallah, A Olczak, J Raftery, N E Chayen, P F Zagalsky and J R Helliwell, (2001) Acta Crystallographica Section D-Biological Crystallography D57, 1219-1229.
3. Cianci, M, Rizkallah, PJ, Olczak, A, Raftery, J, Chayen, NE, Zagalsky, PF and Helliwell, JR, Proc. Natl. Acad. Sci. U. S. A., 2002, 99, 9795-9800.

Poster No. III-4

Human Complement Protein C8γ: The Sole Lipocalin in the Complement System

Sodetz JM, Ortlund E, Parker CL & Lebioda L, Dept. of Chemistry & Biochemistry, University of South Carolina, Columbia, SC, USA

Human C8 is one of five components (C5b, C6, C7, C8, C9) of the cytolytic "membrane attack complex" of complement, or MAC. It contains three genetically distinct subunits, C8α (64kDa), C8β (64kDa) and C8γ (22kDa), which are arranged as a disulfide-linked C8α-γ dimer that is noncovalently associated with C8β. C8α and C8β are homologous and together with C6, C7 and C9 form the MAC family of proteins. By contrast, C8γ is unrelated and is the only lipocalin in the complement system. Distinct roles have been identified for C8α and C8β in the formation and

function of the MAC, however little is known about the role of C8γ. Using a complex of C8α + C8β, it was shown that C8γ is not required for MAC-mediated lysis of erythrocytes nor for killing of sensitive Gram-negative bacteria. Although not essential, C8γ enhances both activities by an unknown mechanism. To gain insight into its function, the structure of C8γ was recently determined by X-ray diffraction to 1.2 Å resolution [(Ortlund, E. *et al*., *Biochemistry* 41, 7030-7037 (2002)]. C8γ displays a typical lipocalin fold that is most similar to neutrophil gelatinase associated lipocalin (NGAL). Both have a hydrophilic entrance and a large hydrophobic cavity at the bottom of the calyx. Access to the lower cavity is restricted in NGAL whereas in C8γ the cavity is accessible and can bind up to three relatively large Xe atoms. Features of the C8γ binding site suggest the ligand is related to a fatty acid, and crystal soaking experiments have shown that lauric acid is capable of binding. Because it enhances C8 hemolytic and bacteriolytic activity, we suggest C8γ may function to stabilize the MAC by binding to a hydrocarbon chain on a membrane-associated lipid. Such chains could be on glycerophospholipids in erythrocyte membranes or the lipid A portion of LPS in Gram-negative bacteria. (NIH GM 042898)

Neutrophil Gelatinase Associated Lipocalin in Health and Disease

Borregaard N & Cowland JB, Rigshospitalet-4042, Department of Hematology, Granulocyte Research Laboratory, Copenhagen, Denmark

Neutrophil Gelatinase Associated Lipocalin (NGAL) is a major protein of human neutrophil granules. It is constitutively expressed in neutrophil precursors at the myelocyte-metamyelocyte stage of maturation and is consequently localized to specific granules with lactoferrin and hCAP18. The expression here is regulated by the myeloid transcription factors PU.1, and C/EBPs, in particular C/EBPε.

It is uncertain whether NGAL is truly constitutitively expressed in other human cells, but it is highly upregulated in a variety of epithelial cells when these are engaged in an inflammatory process and perhaps also in neoplastic transformation.

A549 cells which are derived from type II pneumocytes express NGAL at a low level. This is dramatically upregulated when the cells are exposed to IL-1β. This expression is under control of the NFκB transcription factor. It was initially shown that NGAL binds the bacterial chemotactic factor fMLP but this was later shown to be a non-specific i.e. low affinity binding, not mediated via the lipocalin pocket. Instead, NGAL has been shown to bind the enterobactin siderophore and possibly others. This indicates that NGAL plays an essential role in depriving microorganisms of one of their most important nutritional requirements, Fe^{3+}. Further experimental evidence from a mouse model has shown that the mouse orthologue 24p3 plays a role as an endogenous iron transporter essential for development of the embryonic kidney. It is possible but entirely speculative that NGAL may play similar roles in neoplastic processes.

Lipocalin Allergens

Mäntyjärvi R, Department of Clinical Microbiology, University of Kuopio, Kuopio, Finland

Since 1996 it has become evident that practically all the important respiratory allergens of mammalian origin are lipocalins. Dogs, cattle, horses and the common laboratory rodents are abundant sources of lipocalin allergens and frequent inducers of allergic diseases. Neither the three-dimensional molecular structure nor biologic activity has provided definite clues for the determinants of the allergenicity of lipocalins.

The one common property of all allergens is that they are recognized by the immune system in such a way that an IgE response is initiated. Allergenicity of a protein can be detected only by immunological tests, and therefore it can be defined only in association with a host, a person with an appropriate genetic background. This is illustrated by the studies on allergen epitopes, the sites on the allergen molecule recognized by IgE antibodies or helper T-cells. Information of conformational IgE epitopes and linear T-cell epitopes can be obtained by using synthetic peptides or allergen fragments. Studies with peptide analogues and by site-directed mutagenesis of recombinant allergens have revealed that single amino acids may be critical both for IgE binding and T-cell recognition, but that the effect of amino acid substitutions varies between patients.

We have proposed that the allergenicity of lipocalins may be related to the adaptation of the immune system to the presence of endogenous lipocalins. T-cell epitopes would be recognized by an exposed person's immune system as suboptimal ligands favoring a deviant immune response. These T-cell epitopes may be different in different patients and their identification is a laborious process. On the practical level, however, structural studies and the mapping of both IgE and T-cell epitopes will help in the planning and designing new treatment modalities against allergy.

Poster No. III-5

A Salivary Gland Lipocalin Expressed in Male and Lactating Hamsters with Possible Odorant-Binding and Carrier Function

Srikantan, S & De, PK, Centre for Cellular & Molecular Biology, Hyderabad, India

Hamster **m**ale-**s**pecific **p**rotein (MSP) is abundantly expressed as heterogenously glycosylated and non-glycosylated forms in adult male **s**ub**m**andibular **g**land (SMG; ~40% of soluble proteins). It is copiously secreted in saliva, also endocrine secreted and excreted in urine. Although, absent in normal adult female SMG, its rapid induction to male

levels is seen in SMG, saliva and urine of lactating and gonad- ectomized females, which disappears post-weaning in the former. cDNA cloning and sequence analysis showed that MSP is a lipocalin having maximum identity with **o**dorant/pheromone-**b**inding **p**roteins such as OBP-1/1F of rat nasal mucosa (58%) and aphrodisin of hamster vaginal discharge (39%). Interestingly, hamster nasal mucosa lacks any OBP/MSP-like protein. Ligand binding studies showed that MSP binds fluorescent probe 1-AMA, which was competitively displaced by synthetic odorant IBMP and a hamster odorant DMDS (a male attractant) present in vaginal discharge. SMG of immatures of both sexes express relatively trace levels of MSP. Extensive *in vivo* studies using gonadectomy and/or sex hormone treatments of adult, immature and lactating hamsters revealed unusual and marked repress-ion of MSP by both estrogens and androgens. However, the massive male-specific expression of MSP, seen only after sexual maturity, was due to a developmentally acquired androgen insensitivity and was also unaffected by orchiectomy. On the other hand, the temporary male-like expression during lactation was due to it being a low-estrogen state. The interesting expression pattern and ability to bind specific odorants suggest a role for salivary and urinary MSP (of males and nursing mothers) in chemical communication as a carrier for odor cues known to be present in these secretions. Since, saliva can contact the nasal epithelium and male hamsters invariably lick vaginal discharge of females prior to sex- ual arousal, a role for MSP, as a carrier for odor/pheromonal cues for their perception, is also suggested.

Poster No. III-6

Role of disulfide bonds in the structural stabilization of equine β-lactoglobulin

Ikeguchi M, Kobayashi T, Tokushima A, Yajima T, Saitoh K, Yamada Y, Nakagawa K & Shokita A, Department of Bioinformatics, Soka University, Hachioji, Japan

Equine β-lactoglobulin (ELG) has two disulfide bonds, Cys66-Cys160 and Cys106-Cys119. To address the role of individual disulfide bonds in the structural stabilization, constructed were two mutant proteins in which one of two disulfide bonds is eliminated by substituting Cys by Ala. The circular dichroism spectra have shown that the mutant lacking Cys66-Cys160 (C66A/C160A) assumes a native-like three-dimensional structure whereas the mutant lacking Cys106-Cys119 (C106A/C119A) is not able to assume any specific tertiary structure. These results suggest that Cys106-Cys119 is essential to maintain the native tertiary fold of ELG. This is interesting because some other members of the lipocalin superfamily do not have the disulfide bond corresponding to Cys106-Cys119. Although Cys66-Cys160 is not essential to acquire the native-like tertiary fold, it contributes to the stabilization of the native conformation. C66A/C160A was unfolded at lower urea concentration than wild-type ELG was. The thermal unfolding temperature of C66A/C160A is reduced by 13 degree. The free-energy contribution of Cys66-Cys160 was evaluated to be 1.8 to 3.1 kcal/mol from the difference in the free energy of unfolding between C66A/C160A and wild-type ELG. This contribution is much smaller than that theoretically expected from the chain entropy increase in the randomly coiled state, suggesting that some residual structures are present both in the thermally unfolded state and in the urea unfolded state.

Poster No. III-7

NMR interaction studies of chicken liver basic fatty acid binding protein

Catalano M, Ugolini R, Ragona L, Luppi M & Molinari H, Università degli Studi di Verona Dipartimento Scientifico e Tecnologico, Verona, Italy

Chicken liver fatty acid binding protein (Lb-FABP) belongs to the basic type fatty acid binding proteins, a novel group of proteins isolated from liver of different non mammalian species whose structure is not known. Based on the high sequence and structural similarity with an orthologous protein, ileal lipid binding protein, we have suggested that bile acids, rather then fatty acids, may be the putative ligands (1).

Here we report the NMR solution structural studies of the recombinant apo Lb-FABP and of its complex with palmitic and chenodeoxycholic acid, a primary bile acid in humans.

The ^{1}H and ^{15}N resonance assignments for apo and holo Lb-FABP have been determined by 2D homonuclear and 3D heteronuclear NMR spectroscopy.

The overall fold of the apo-structure at pH 7 is common to other proteins of the family and consists of ten antiparallel beta-strands organised in two nearly orthogonal beta-sheets with two alpha-helices closing the protein cavity where hydrophobic ligands are bound.

The stoichiometry and the conformational properties of the holo Lb-FABPs (complexed with palmitic and bile acids) will be discussed in light of the data reported for the structurally similar apo and holo ileal lipid binding protein (2). This comparative analysis will be extended to the beta-lactoglobulins.

1 F. Vasile, Laura Ragona, M. Catalano, L. Zetta, M. Perduca, H. Monaco and H. Molinari
J. Biomol. NMR **2003**, *25*, 157-160
2 Lucke C, Zhang F, Hamilton JA, Sacchettini JC, Ruterjans H. *Eur. J. Biochem.* **2000,** *267*, 2929-38

Poster No. III-8

The binding-status of lipocalins (*holo* vs. *apo*) determines the specificity for the RBP receptor

Redondo C, University of Leeds, Leeds, UK

Several studies have demonstrated that the interaction of Retinol Binding Protein (RBP) with its cell surface receptor is mediated by the loops at the mouth of the ligand-binding site. To confirm that RBP's CD loop is the main motif responsible for its interaction with the receptor, the loop was grafted into the Major Urinary Binding protein (MUP) and the chimaera compared with RBP for its ability to interact with membrane preparations of HEK 293 cells.

Using Surface Plasmon Resonance (SPR), binding was assayed with partially solubilised membrane preparations of HEK 293 cells, using *holo* and *apo* forms of RBP and MUP_{CD} coupled to NTA sensorchips. Experiments were conducted by flowing a saturable amount of ligand over the sensorchips [2-isobutyl-3-methoxypyrazine (IBMP) and all-trans-retinol, for MUPcd and RBP, respectively] prior to the injection of solubilised membranes. *Holo* and *apo* forms of WT-MUP were also included in the experiments and membranes prepared from red blood cells (ghosts) were used as a negative control (since they were previously described as not exhibiting a receptor for RBP).

The results obtained confirm the preliminary observations that the CD loop is indeed responsible for the specificity of binding to the membrane receptor present in HEK 293 cells. This was evident from the observation that both *holo* forms of RBP and MUP_{CD} could bind to the membranes, but *holo*-WT MUP could not. A novel interesting finding was that none of the *apo*-forms of the lipocalins studied could bind to the membranes, suggesting that the presence of the ligand determines a conformation that is recognised by the receptor, allowing complex formation. Moreover, the conformational effects on the CD loop were equivalent even though the core of the lipocalin was different, perhaps suggesting a related conformational coupling mechanism. It is likely that the receptors for RBP and MUP are different, since WT MUP unlike MUP_{CD}, could not bind to HEK 293 cells.

Poster No. III-9

Bovine and porcine beta-lactoglobulins: interaction and solvation studies

Ragona L, Catalano M, Fogolari F, Marini A, Ugolini R, Zetta L & Molinari H, Istituto per lo Studio delle Macromolecole, CNR, Milan, Italy

The study of homologous proteins belonging to the same family can provide fundamental insights into the determinants of important properties such as folding mechanism and mode of binding. With this aim we started the comparative analysis of two beta-lactoglobulins, from bovine and porcine species, sharing 80% similarity and different aggregation, folding and binding properties .

The present work reports our recent results on ligand interaction and solvation properties of beta- lactoglobulins, as deduced from NMR measurements and long timescales MD simulations.

Bovine beta-lactoglobulin (BLG), isolated from milk, shows endogenously bound fatty acids, differently from porcine (PLG) and equine that neither have fatty acids bound nor are able to bind them at physiological pH. The observed behaviour is discussed in light of the so called Tanford transition involving the opening of the EF loop at the beta-barrel open end.

Interactions of proteins with other molecules can ultimately be ascribed to their surface features. BLG surface accessibility has been investigated by a combined NMR analysis of water-protein Overhauser effects and paramagnetic perturbation profiles induced by soluble spin-labels. This approach seems to be reliable not only for distinguishing between buried and exposed residues but also for finding molecular locations where a network of more ordered waters covers the protein surface. This approach, which makes use of a series of 2D ePHOGSY experiments, has been complemented by long timescales MD simulations in explicit water, to sort out the water motions on different time-scales.

Poster No. III-10

Comparison between unfolding/refolding of bovine and porcine *Odorant Binding Proteins*

Parisi, M, Mazzini, A, Sorbi, RT, Grolli, S, Ramoni, R & Favilla, R, University of Parma – Department of Physics, Parma, Italy

Our studies are focused on the equilibrium unfolding of two Odorant Binding Proteins (bovine and porcine OBP), which are in a different aggregation state at neutral pH: bOBP is a dimer stabilized by domain swapping, pOBP is a monomer. In both cases unfolding is induced with guanidinium hydrochloride (GdnHCl) and is completely reversible in terms of recovered structure and function. The process is followed by monitoring changes of protein intrinsic fluorescence and circular dichroism. Both transition curves are similar and have a protein concentration independent midpoint. However, the analysis of the equilibrium transition is different. In the case of dimeric bOBP, this result implies a sequential ($N_2 \leftrightarrow 2N \leftrightarrow 2D$ or $N_2 \leftrightarrow D_2 \leftrightarrow 2D$), rather than a concerted ($N_2 \leftrightarrow 2D$) unfolding process, i.e. the involvement of an intermediate state. However, no intermediate is detected in unfolding process, implying it must be present in very small amounts or have optical properties similar to either the native or the denaturated protein. The thermodynamic transition parameters are thus obtained using a simple two state model ($N \leftrightarrow D$) for both proteins ($\Delta G°_{un,w}$ =5.0 mol^{-1} kcal, m = 1.9 mol^{-1} kcal M^{-1} ,$C_{1/2}$ = 2.6 M for bovine OBP and $\Delta G°_{un,w}$ = 4.50 mol^{-1} kcal, m= 1.84 mol^{-1} kcal M^{-1}, $C_{1/2}$ = 2.45 M for porcine OBP). For both OBPs the presence of the ligand dihydromyrcenol has a

stabilising effect against unfolding by GdnHCl and the transition midpoints shift towards greater concentration of denaturant ($C_{1/2}$ = 4.6 M for bOBP, 3 M for pOBP). On the contrary, refolding is different for the two proteins. While pOBP refolds completely in a day, renaturation of bOBP presents hysteresis at medium-long times. This allows us to detect a native-like monomeric intermediate whose stability is probabily due to a slow step of dimerisation by domain swapping.

Poster No. III-11

Optimization of the solubility properties of recombinant human ApoD

Nasreen A & Skerra A, Institute of Biological Chemistry, Technical University Munich, Freising-Weihenstephan, Germany

Apolipoprotein D is an important member of the lipocalin structural family, which was discovered as a peripheral subunit of the high density lipoprotein particle. It is also abundant in various tissues, e.g. liver, kidney, brain, and several body fluids. However, its biological function remains largely speculative. It was shown that ApoD complexes progesterone and arachidonic acid as physiological compounds [1]. Unfortunately, there is only a hypothetical structural model available [2], which was based on sequence homology with the bilin-binding protein (BBP), whose crystal structure is known. Therefore, experimental elucidation of the three-dimensional structure of ApoD is desirable. ApoD can be expressed as soluble recombinant protein in *E. coli* via secretion into the bacterial periplasm [1], whereby the free Cys116 residue has been replaced by Ser. The protein was purified via the *Strep*-tag II and ligand binding was demonstrated by fluorescence titration. However, the bacterially produced protein exhibits strong tendency to aggregate in solution and to adsorb to surfaces. For example, the recombinant ApoD cannot be recovered from a gel filtration column in a functional state.

The pronounced aggregation tendency and the corresponding restriction in the choice of purification procedures hamper crystallization experiments. Hence, we mutated exposed hydrophobic side chains of ApoD in a systematic manner and determined the corresponding solubility properties. As a result, we describe one mutant which has the following features: (a) improved yield upon expression in *E. coli*; (b) elution as a monomeric protein by gel filtration; (c) retained affinity for progesterone as a ligand. This engineered ApoD appears to be promising for structural studies.

1. Vogt, M. & Skerra, A. (2001). *J. Mol. Recognit. 14*, 1-8.
2. Peitsch, M. C. & Boguski, M. S. (1990). *New Biol. 2*, 197-206.

Poster No. III-12

Recombinant wild type and mutated human a_1-microglobulin – purification procedure and general physicochemical properties

Kłapyta A[a], Osmark P[b], Allhorn M[b], Åkerström B[b] & Wasylewski Z[a], [a]Department of Physical Biochemistry, Faculty of Biotechnology, Jagiellonian University, Poland, [b]Department of Molecular Biology, Biomedical Center, Lund University, Sweden

Production of the recombinant wild type and several mutants of human a_1-microglobulin (a_1m), purification procedure and general physicochemical characterisation is described.

The human lipocalin a_1m, found in plasma and tissues, is involved in heme-metabolism. A covalently linked yellow-brown chromophore has been shown to be due to modifications of the side-chains at Cys34, K92, 118 and 130. DNA coding for eight variants of human a_1m were constructed and cloned into a pET vector. The resulting proteins were wild type (wt), C-mutant (Cys34→Ser), K_3-mutant (Lys92→Thr, Lys118→Thr and Lys130→Thr) and K_3C-mutant (Cys34→Ser, Lys92→Thr, Lys118→Thr and Lys130→Thr), all four mutants constructed with or without the C-terminal tetrapeptide, LIPR. The constructs contained an N-terminal eight-histidine tag followed by an enterokinase site. Expression of the recombinant proteins was performed in *E. coli*. Inclusion bodies formation required extraction of the proteins with high concentrations of guanidinium chloride. After preliminary purification in denaturing conditions on a nickel-agarose column, the proteins were successfully folded. Second purification in native conditions by IMA chromatography resulted in high yields of about 90-95% pure proteins, as estimated by SDS-PAGE. Proper folding of all recombinant $a_1$ms was verified using radioimmunoassay. Far UV circular dichroism spectra revealed no significant differences between the recombinant wt and mutated a_1m and their shapes are analogical to the one obtained for the human protein. UV-VIS absorbance and fluorescence spectroscopy showed the presence of small but significant amounts of chromophore on wt-α_1m, but much less than on human plasma and urinary α_1m.

Poster No. III-13

Characterisation of lipocalins and their receptors

Burke, B, University of Leeds, Leeds, UK

The ability of lipocalins to interact with a variety of molecules has been well reported in the literature. Much effort in this laboratory has been spent studying the interaction of RBP and its receptor resulting in identification of key regions for interaction. Further work demonstrated successful transfer of receptor binding specificities from one lipocalin to another. More recently work has been focused on production of recombinant VEG in *E.coli* in order to study its role in cellular uptake of ligands such as retinol. The latter construct is expressed as a soluble hexahistidine fusion protein and

its functionality has been tested by measuring binding to ^{3}H-retinol. Our immediate concern is examination of the specificity of the interactions of VEG which will be tested using a variety of binding experiments in conjunction with site directed mutagenesis with a view to investigating any uptake processes uncovered.

Poster No. III-14

Heating of β-lactoglobulin A at high-temperature in closed system

Wada R & Kitabatake N, Kurashiki Sakuyo University, Faculty of Food Culture, Kurashiki-shi, Okayama-ken, Japan

Heating of food at high-temperature (>100°C) is one of the most commonly used processes in closed system food such as canned food, retort food, extrusion food etc. and the effect on the conformational changes of proteins might be expected. Changes of secondary structure and aggregation of β-lactoglobulin A (βLG A) and βLG A modified with *N*-ethylmaleimide (NEM-βLG A) were investigated using circular dichroism (CD) spectroscopy and Fourier transform infrared spectroscopy (FT-IR). Far-UV CD spectra of NEM-βLG A at pH 7.5 and βLG A at pH3.0 shifted the spectra from 120 to 130°C assigned to β-sheet structure. FT-IR spectra of βLG A at pH 2.0 assigned to β-sheet structure also showed the change at the same range of temperature. It suggested that the change of this secondary structure occurred in intra molecular and this change was characteristic of temperature.

Poster No. III-15

Characterization of Wildtype and C34S-Human Protein HC: Differences in Amount of Fluorescent Charge-Heterogeneous Chromophore and in Complex-Forming Capacity

Solís J, Calero M, Grubb A, Abrahamson M, Medina M, Gavilanes K, Schneider & Méndez E, Department of Clinical Chemistry, Lund University, Lund, Sweden

Protein HC, also named a1-microglobulin, is a 30 kDa-glycoprotein unique among the lipocalin superfamily, since it is covalently linked to a fluorescent yellow-brown chromophore displaying extensive charge-heterogeneity. It has been suggested that the chromophore is mainly attached to the Cys 34 and that it is involved in the covalent complex-formation with IgA, IgA-HC being the quantitatively dominating blood plasma form of protein HC. In the present study, recombinant wildtype human protein HC (rwtHC) was produced, as well as a variant with cysteine-34 replaced by serine (rC34SHC), by expression in baculovirus-infected insect cells. Biochemical and physicochemical characterization showed similar results for rwtHC, rC34SHC and native protein HC isolated from human urine (uHC). The molecular masses of rwtHC and rC34SHC were slightly lower than that of uHC due to differences in glycosylation. CD studies suggested an almost identical secondary structure for rwtHC, rC34SHC and uHC. Surprisingly, the absorbance and fluorescence properties of the chromophore of rwtHC were virtually equal to those of the chromophore of uHC. The marked charge-heterogenity of uHC was also displayed by rwtHC as demonstrated by agarose gel electrophoresis. In contrast, spectral analysis of rC34SHC showed its chromophore amount to be only about 30% of that of uHC and rwtHC. Furthermore, rC34SHC migrated upon agarose gel electrophoresis as two charge-homogeneous bands, demonstrating the crucial role of Cys34 in chromophore formation. In addition, the complex-formation of the recombinant proteins with IgA were studied. rwtHC could form an i*n vitro* complex with IgA but rC34SHC could not, indicating the relevance of Cys34 in complex formation. In conclusion, Cys 34 of protein HC plays a crucial role for generation of the chromophore and protein-complexes of this lipocalin protein.

BENZON SYMPOSIUM No. 50

THE LIPOCALIN PROTEIN SUPERFAMILY

AUGUST 24-28, 2003, COPENHAGEN, DENMARK

Organizing committee:
Bo Åkerström (Lund), Darren Flower (Compton), Jean-Philippe Salier (Rouen) and Niels Borregaard (Copenhagen)

Abstracts - THURSDAY, August 28, 2003

Anti-inflammatory properties of specific glycoforms of alpha-1-acid glycoprotein
van Dijk W, Department of Molecular and Cellular Biology & Immunology, VU medical center, Amsterdam, The Netherlands
Alpha-1-acid glycoprotein (AGP) is a human plasma protein that belongs to the group of positive acute-phase proteins that are produced by the liver. It is also regarded to be a member of the lipocalin family and has the ability to bind and carry numerous basic and neutral lipophilic drugs from endogenous (steroid hormones) and exogenous origin. An interesting feature of AGP is its anti-TNF effect that may rely in its ability to reduce the capillary permeability.

Studies *in vitro* as well as in animals have shown that AGP also has various anti-inflammatory properties which are dependent on the composition of its glycans. AGP is a highly glycosylated molecule containing five N-linked glycans comprising 45 % of its apparent molecular weight of 43 kD. The N-linked glycans display a microheterogeneity with respect to their extent of branching (di-, tri- and tetraantennary glycans), degree of fucosylation and sialylation (*i.e* the presence of sialyl Lewis^X groups). This is reflected in the presence of various AGP-glycoforms in plasma. Inflammatory reactions induce strong increases in plasma levels of sialyl Lewis^X containing AGP-glycoforms as well of AGP-glycoforms with two or more diantennary glycans. These increases have been shown to be a consequence of cytokine-induced changes in the hepatic glycosylation of AGP. Remarkably, specific anti-inflammatory properties of AGP have been shown to reside in these AGP-glycoforms. For instance, diantennary-containing AGP-glycoforms can inhibit CD3-induced proliferation of lymphocytes, and sialyl Lewis^X containing human AGP-glycoforms can ameliorate neutrophil- and complement-mediated injuries in rat models. It is not known yet, to what extent the AGP apoprotein is involved in these reactions. The results suggest that changes in plasma concentration of specific AGP-glycoforms will contribute to the beneficial effects of the hepatic acute-phase reaction.

An Update on the Immunocalins, the Immunomodulatory Lipocalins
Lögdberg L, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, ECLH, Atlanta, GA, USA
Reviewing the growing lipocalin literature, in late 1999, with particular attention to potential interactions[between lipocalins and the immune system, we focused on a subset of seven genetically linked (gene cluster in the q32-34 region of chromosome 9) lipocalins (α_1-acid glycoprotein, α_1-microglobulin, glycodelin, neutrophil gelatinase-associated lipocalin, complement factor γ-subunit, tear prealbumin, and prostaglandin D synthase). All appear to have functional properties consistent with functional molecules of the innate immune system (protective immunoregulatory/anti-inflammatory/antimicrobial effects; 5 are involved with the acute phase response). We proposed that they and other putative family members, may form a functional lipocalin subfamily that we provisionally named the immunocalins.

Now, 3 ½ years later, the lipocalin literature (PubMed-articles using the word lipocalin) has roughly doubled and we again have conducted an up-to-date review of this subfamily, highlighting major developments. One striking new finding is the interaction of at least two of the members (α_1-microglobulin and neutrophil gelatinase-associated lipocalin) with iron-metabolism, such as organogenesis related iron-delivery and heme-binding and –degradation. This suggests multiple new mechanisms for lipocalin-based innate immunoprotection, including tissue protection against oxidative stress and antibacterial iron depletion.

EX-FABP in differentiation and pathology
Descalzi Cancedda F[1,2], Di Marco E[2], Gentili C[2], Pagano A[2], Cermelli S[2], Zerega B[2] & Cancedda R[2,3] Consiglio Nazionale delle Ricerche[1], Istituto Nazionale per la Ricerca sul Cancro[2], University of Genova[3], Genova, Italy
Ex-FABP is a chicken extracellular fatty acids binding lipocalin, expressed in embryonic hypertrophic cartilage, in newly formed muscle fibres, in myocardium in developing heart, in liver and in granulocytes. Ex-FABP behaves as an acute phase protein. Expression of Ex-FABP is enhanced by inflammatory agents, such as bacterial endotoxin LPS

and interleukin 6, in "in vitro" differentiating hypertrophic chondrocytes, in forming myotubes and in cultured cardiomyocytes. Also fragments of embryonic liver in culture show increased expression of Ex-FABP by treatment with LPS. Non steroidal antiinflammatory agents repress the synthesis of the protein both when expressed during cell differentiation and when induced by LPS. In adult animals Ex-FABP is expressed in articular cartilage of osteoarthritic chickens and in the lesion areas of dyschondroplastic chicken bones. We have proposed that Ex-FABP is a stress protein, expressed both during development in tissues where active remodelling is taking place and as part of an acute phase response in tissues under pathological conditions. In order to investigate Ex-FABP biological role, proliferating chondrocytes were transfected with an expression vector carrying antisense oriented Ex-FABP cDNA. Following Ex-FABP suppression, we observed extensive cell death and a strong inhibition of cell proliferation and differentiation. When chondrocytes were transfected with an antisense oriented Ex-FABP cDNA under the control of a Doxycycline-inducible promoter, Ex-FABP down-modulation after Doxycycline induction increased the number of apoptotic cells. Myoblasts transfected with the same expression vector carrying antisense oriented Ex-FABP cDNA showed extensive cell death and impaired myotube formation. Microinjection of chicken embryos with antibodies against Ex-FABP showed that 70% of chicken embryo died and the target tissue was the heart. We suggest that Ex-FABP acts as a constitutive survival protein and that its expression and activation are fundamental to escape cell death in stress conditions. Moreover the protein is part of a lipocalin cluster whose members are coexpressed.

Glycodelin: A major lipocalin protein of the reproductive axis with diverse actions in gamete binding, immune reactions and differentiation

Seppälä M, Koistinen H, Mandelin E, Yeung WSB, Koistinen R, Departments of Clinical Chemistry, and Obstetrics and Gynecology, Helsinki University Central Hospital, 00029 HUS, Helsinki, Finland

Glycodelin is a 28 kDa glycoprotein of the female and male reproductive axes, with structural homology with beta-lactoglobulins from various species. Depending on glycosylation, glycodelin appears in various isoforms, three of which have been isolated and characterized. Glycodelin-A (GdA) is the major secretory glycoprotein in endometrium, GdF (or zona-binding inhibitory factor-1, ZIF-1) in follicular fluid, and GdS in male seminal plasma. In the uterus, GdA is progesterone-regulated and it is secreted into uterine luminal cavity by secretory/decidualized endometrial glands. GdA and GdF potently and dose-dependently inhibit human gamete interaction by binding on the human sperm. GdA shares one of the two binding sites of GdF on spermatozoa. Deglycosylation of GdF results in loss of its zona-binding inhibitory activity, and differently glycosylated GdS from seminal plasma has no inhibitory effect at all. These results demonstrate the importance of glycosylation for biological activity of glycodelin isoforms. GdA is absent from endometrium during the fertile window and, in an ovulatory cycle, glycodelin appears on day LH+5 and its secretion remarkably increases until the onset of menstruation, unless pregnancy ensues. During pregnancy, glycodelin secretion continues to increase until the 10^{th} week, thereafter decreasing. Use of progestagen-related contraception is accompanied by glycodelin secretion over the fertile window, likely contributing to contraceptive activity. Glycodelin also has immunosuppressive activity. Thus, the recognition mechanisms in the immune and reproductive systems may have converged, while the relationship between glycosylation and immunosuppressive activity remains to be investigated. The high glycodelin concentration at the fetomaternal interface and its NK cell inhibitory activity suggest a role in fetomaternal defense mechanisms during the window of implantation and pregnancy. Glycodelin is related to epithelial differentiation, and transfection experiments suggest that glycodelin may play a role in glandular morphogenesis. This disposition may have bearance on patients with cancer.

Poster No. II-5

Glycodelin A: An Immunocalin with Apoptotic Activity

Karande AA, Mukhopadhyay D, Jayachandran R & SundarRaj S, Department of Biochemistry, Indian Institute of Science, Banglore-560 012, India

Glycodelin A [GdA], a member of the lipocalin family is secreted by the endometrium during the secretory phase of the menstrual cycle and pregnancy under progesterone regulation. The temporal and spatial expression of GdA in the female reproductive tissue indicates that the protein is associated with establishment and maintenance of pregnancy and ample evidence exists in literature to suggest that GdA modulates the maternal immune system from attacking the fetal allograft.

Recent data from our laboratory have shown that GdA exerts its 'immunomodulatory' activity by inducing apoptosis in T cells. This was found to be through activation of the mitochondrial pathway and not through engagement of death receptors. The role of glycosylation in this activity of the molecule was addresssed by site-directed mutatagenesis studies involving the relevant asparagine residues.

Another form of glycodelin (glycodelin S, [GdS]) is present in the seminal fluid. The two isoforms of this protein are reported to be identical except for their glycosylation. Experimental data in our laboratory have shown that GdS lacks apoptotic activity. We also observed that the glycan structures on GdA do not induce apoptosis on their own, suggesting the possibility of differential folding attributed to differential glycosylation patterns. The folding status of the two isoforms were analysed and compared. While GdA appears to have a stable structure, GdS was found to be relatively unstable.

Poster No. IV-2

Construction of Anticalins as Antagonistic Agents for the Target-specific Intervention in Autoimmune Diseases

Haug G & Skerra A, Institute of Biological Chemistry, Technical University of Munich, Freising-Weihenstephan, Germany

T-cell activation is not only dependent upon signals provided through the interaction of an antigen-specific T-cell receptor with its cognate peptide presented by histocompatibility complex class II but also on several costimulatory signals. The B7 family of costimulatory molecules, mainly B7.1 (CD80) and B7.2 (CD86), together with their corresponding T cell coreceptors, CD28 and CTLA-4, constitute the most well characterized pathway up to now, opening possibly generic strategies for therapeutic intervention in immune-mediated diseases [1]. Blockade of this pathway with a soluble CTLA-4-Ig fusion protein, for example, induced anergy of antigen-specific T-cells *in vitro*. Nevertheless, the generation of an artificial receptor protein with improved tissue penetration and pharmacokinetics would be desirable.

Lipocalins with an appropriately engineered target specificity could provide a suitable protein scaffold for this purpose [2]. In this study we demonstrate the cloning, bacterial production, and one-step purification of the extracellular domain of murine B7.1. A random library based on the bilin-binding protein was screened for affinity towards this protein target via phage display and colony screening. Thus led to the identification of several lipocalin variants recognizing the recombinant mB7.1 with pronounced affinity and specificity, so-called anticalins [3]. These provide a promising starting point in order to evaluate the capability for *in vitro* competition with CTLA-4 and for attempts to achieve immunomodulation in animal models.

[1] Carreno, B. M., Collins, M. (2002) *Annu. Rev. Immunol.* 20, 29-53.
[2] Skerra, A. (2000) *Biochim. Biophys. Acta* 1482, 337-350.
[3] Beste, G., Schmidt, F. S., Stibora, T., Skerra, A. (1999) *Proc. Natl. Acad. Sci. USA* 96, 1898-1903.

Poster No. IV-3

Unique T cell immunoregulatory properties of the lipocalin placental protein 14

Rachmilewitz, J, Weber, MC, Borovsky, Z, Mishan-Eisenberg, G, Riely, GJ & Tykocinski, ML, University of Pennsylvania, Philadelphia, PA, USA, and Hadassah Medical Center, Jerusalem, Israel

The lipocalin placental protein 14 (PP14; glycodelin) is present at high levels in decidualized endometrium, amniotic fluid, and the serum of pregnant women. It is also found in abundance in male seminal fluid, as well as in cells of the megakaryocytic lineage and in certain tumor types. In exploring this lipocalin's unique T cell immunoregulatory properties, we have established that PP14 inhibits T cells directly, by desensitizing T cell receptor (TCR) signaling and thereby elevating TCR activation thresholds. A series of further studies have permitted us to formulate a hypothesis to explain this T cell immunomodulatory effect. According to this hypothesis, PP14 promotes the dephosphorylation of TCR-induced phosphoproteins via CD45, a major protein tyrosine phosphatase receptor on T cell surfaces. Our data further suggest that PP14 engages the CD45 glycoprotein as a lectin and thereby promotes CD45's dephosphorylating activity, possibly by interfering with its timely exit from APC:T cell contact sites. This proposed mechanism of action draws parallels between PP14 and galectin-1, another CD45-binding protein, and may explain our earlier finding that α_2-macroglobulin amplifies PP14's inhibitory activity, perhaps by promoting the formation of higher-order lattices. Taken together, these data suggest a model wherein PP14, acting in a lectin-like mode, enters and alters the organization of immune synapses, thereby negatively regulating proximal TCR signaling. This mode of action is consistent with a putative role for PP14 in the amelioration of certain cell-mediated autoimmune diseases during pregnancy. (Supported in part by NIH R01 AI38960 and NIH R03 TW00614801).

Poster No. IV-4

α_1-Acid Glycoprotein (AAG), A Possible Carrier of Sialyl Lewis X (SLEWIS X) Antigen in Colorectal Carcinoma

Croce MV, Sálice VC & Segal-Eiras A, Center of Basic and Applied Immunological Research (CINIBA), Faculty of Medical Sciences, National University of La Plata, La Plata, Argentina

Different carbohydrate antigens such as sLewis x have been associated with metastatic process in colorectal cancer while diverse putative carriers to these carbohydrate antigens have been proposed although results are not conclusive. On the other hand, it has been reported that AAG associated with inflammatory tissues expresses sLewis x. Therefore, we developed this research for: 1- to detect AAG and sLewis x in colorectal malignant, benign and normal samples; 2- to isolate AAG from colorectal cancer and 3- to study its immunoreactivity with anti-s Lewis x monoclonal antibody (MAb). Materials and methods: tissue samples from 32 colorectal cancer, 15 adenomas and 24 normal colorectal biopsies were included. MAbs directed to sLewis x (KM93) and to AAG (SIGMA) were employed. Expression of AAG and sialyl Lewis x was studied by immunohistochemistry (IHC) following standard procedures with antigenic retrieval. Isolation approach: AAG was precipitated with ammonium sulphate followed by immunoprecipitation with anti-AAG polyclonal antibody. The immune complex formed was isolated by affinity chromatography in protein A-Sepharose CL-4B and further eluted with glycine-HCl buffer pH 3.8. Fractions eluted were studied by SDS-PAGE

and Western-blot. Data were statistically analyzed by means of Principal Component Analysis with Kendall correlations. Results: by IHC, AAG was expressed in 40% malignant, 33% benign and 38% normal samples while sLewis x was detected in 30% malignant samples, 40% benign and in a few normal samples. Malignant samples showed a non-apical pattern of expression comprising the whole cell (cytoplasm and membrane); in normal and benign samples the reaction was restricted to the apical side. In malignant specimens, a statistically significant positive correlation was found between AAG and sLewis x (π=0.1902) expression. By Western blot employing anti-AAG MAb and sLewis x MAbs, fractions isolated from malignant samples showed a band at 45kD. Conclusion: AAG may constitute a possible carrier of sLewis x in colorectal cancer.

Anticalins: engineered lipocalins with antibody-like ligand-binding properties

Skerra A, Technical University Munich, Freising-Weihenstephan, Germany

Lipocalins provide a promising scaffold [1] for the generation of novel ligand-receptor proteins via combinatorial protein design. Despite low mutual sequence homology they share a circularly closed eight-stranded anti-parallel b-sheet as structural motif. At its open end this b-barrel supports four loops, which form the entrance to the binding pocket. The loops exhibit large conformational differences between individual lipocalins and give rise to the variety of natural ligand specificities. The protein architecture is thus reminiscent of immunoglobulins with their hypervariable loops on top of a rigid framework. However, with respect to antibodies – or their recombinant fragments – lipocalins provide several practical benefits because they are merely composed of a single polypeptide chain, have a much smaller size, and their set of four loops can be more easily manipulated at the genetic level.

Initially, we set out to reshape the ligand pocket of the bilin-binding protein from *Pieris brassicae*, thus creating artificial ligand-binding proteins termed "*anticalins*" [2,3]. A molecular library was prepared by subjecting 16 amino acid positions within the four loops to random mutagenesis, followed by panning with different immobilized compounds via phage display. Lipocalin variants with high affinties and specificities for organic molecules, like fluorescein or the widely applied digoxigenin group, peptides, and even proteins – as potential medical disease targets – have thus been generated. Consequently, *anticalins* should provide useful molecular tools in biotechnology, bioanalytics, and medicine.

1. Skerra, A. (2000) *Biochim. Biophys. Acta* 1482, 337-350.
2. Beste, G., Schmidt, F.S., Stibora, T. & Skerra, A. (1999) *Proc. Natl. Acad. Sci. USA* 96, 1898-1903.
3. Skerra, A. (2001) *Rev. Mol. Biotechnol.* 74, 257-275.

Research Beyond the Lipocalin Protein Family: Future Horizons

Flower DR, Edward Jenner Institute for Vaccine Research, Newbury, UK

In the words of Alfred North Whitehead, the British mathematician and philosopher, "Seek simplicity, and then distrust it." At present, human understanding of biology is primarily phenomenological, but in the post-genomic era this is giving way to a profoundly deeper comprehension based on our physical chemical understanding of individual molecular events. Our appreciation of life is changing from a top-down to a cohesive, integrated, bottom-up view. The lipocalins exemplify this well. We understand physical and structural aspects of the family rather better than we understand function. The lipocalins are characterised by three types of molecular recognition event: the binding of small molecules within the intra-calyx cavity, the formation of macromolecular complexes, and the binding to receptors. These recognition properties manifest themselves in remarkably diverse ways: a diversity mirrored in their in their highly divergent sequences, their wide phyletic spread, and, where known, in apparent function. Moreover, the lipocalins, fatty acid binding proteins, Avidins, and Triabin form a large, diverse superfamily: the calycins. The size, evolution, and even the existence, of the calycins remains controversial. A number of receptors for lipocalin family members have also now been identified. Biotechnology offers the opportunity to make modified lipocalins with modified specificities for ligand and macromolecule binding. In this talk, I will touch upon some of the implications of all these aspects. While much concerning lipocalin function remains to be discovered, extant work suggests that they fulfil many critical functions, as immune mediators for example, and the proper characterization of lipocalin receptors will allow the development of novel agonists and antagonists of lipocalin function. Long term the opportunities thus created by researching lipocalin interactions outside the family may yet fundamentally alter the apparent importance of the family. These are, indeed, exciting times for lipocalin research.

Poster No. IV-1

LIMR, the prototype of a novel family of endocytic receptors, is essential for cellular internalization of Lipocalin-1

Wojnar P, Lechner M, Merschak P & Redl B., Department of Molecular Biology, University of Innsbruck, Innsbruck, Austria

Apart from the specific function of Human lipocalin-1 (Lcn-1, also called tear lipocalin or VEG) in stabilizing the lipid film of human tear fluid, it is suggested to act as a physiological scavenger of potentially harmful lipophilic compounds, in general. To characterize proteins involved in the reception, detoxification, or degradation of these ligands, a cDNA phage-display library from human pituitary gland was constructed and screened for proteins

interacting with Lcn-1. Using this method an Lcn-1 interacting phage was isolated that expressed a novel human protein. Molecular cloning and analysis of the entire cDNA indicated that it encodes a 57-kDa membrane protein, LIMR (lipocalin-1 interacting membrane receptor). Biochemical investigations confirmed the LIMR/Lcn-1 interaction. The membrane location of LIMR was verified by immunocytochemistry and Western blot analysis of membrane fractions of human NT2 cells. In a next step, we investigated the physiological role of LIMR using an antisense gene knock-out technology in the human NT2 cell line and found this protein to be essential for mediating internalization of Lcn-1 in NT2 cells. Since sequence and structure analysis indicated that proteins similar to LIMR are present in several organisms and at least two closely related orthologous are found in human and mouse, we suggest LIMR to be the prototype of a new family of endocytic receptors, which are topographically characterized by nine putative transmembrane domains and a characteristic large central cytoplasmic loop.

Poster No. IV-5

Lipocalin-Type Prostaglandin D Synthase and Bilirubin-Related Components in Cerebrospinal Fluid of Patients with Neurological Disorders

Hiraoka A (a), Seiki K (b), Oda H (b), Eguchi N (c), Urade Y (c), Tominaga I (d) & Hori K (d), (a) Kyorin University School of Health Sciences, Hachioji, Tokyo, (b) Maruha Corporations, (c) Osaka Bioscience Institute, (d) Shimohusa National Sanatorium

We measured the concentrations of lipocalin-type prostaglandin D synthase (L-PGDS), bilirubin (BR) and its oxidized products, biopyrrins (BP), in cerebrospinal fluid (CSF) of patients with various neuro- logical disorders. L-PGDS in CSF increased mainly in patients who were recovering from the organic damage in the central nervous system (CNS), as well as in those with pathological brain atrophy. The CSF level of BR was higher in patients with neurodegenerative diseases accompanied by pathological brain atrophy, while that of BP was remarkably elevated in cerebral infarction patients removing from the acute ischemia state. A significantly positive correlation was observed between the CSF levels of L- PGDS and BR in neurodegeneartive disease patients, as well as between those of L-PGDS and BP in cerebrovascular disease patients. In a Guillain-Barre syndrome patient from whom CSF samples were taken 7 times during the period of hospital treatments, the greatest values of the BP and BR concentrations in the 7 samples, which were found in the acute phase and during the recovery phase, were accompanied by the highest and the second highest L-PGDS levels, respectively. These results suggested that the production of L-PGDS as a carrier of BR and BP in the CNS is accelerated under various pathological conditions in association with the oxidative stress.

Poster No. IV-6

Ex-FABP, a lipocalin associated to hearth development and cell survival

Gentili C., Tutolo G., Zerega B., Cancedda R., Descalzi Cancedda F.& Di Marco E., Istituto Nazionale per la Ricerca sul Cancro, University of Genoa, CNR. Consiglio Nazionale delle Ricerche. IBFM, Genova, Italy

Ex-FABP, an extracellular fatty acid binding lipocalin, is physiologically expressed during chicken embryo development and in acute phase response due to pathological conditions. The synthesis and localization of Ex-FABP were detected in cartilage, bone, in newly formed muscle fibers, heart, liver and granulocytes. In this work we observed gene and protein expression during different stage of embryo development by whole mount in situ hybridization and immunoistochemistry analysis. Tissue distribution studies revealed that at early stage (3-4 day) the level of transcript was high and diffuse in all embryo body, whereas the protein was localized at high level in heart and at low level in cartilage and muscle. During the embryo development we observed a modulation of Ex-FABP expression and at 11 day of development, the protein is mainly expressed in cartilage and muscle and decreases in heart. Moreover we observed that Ex-FABP mRNA increases dramatically in embryos during acute phase response induced by endotoxin LPS injection and mostly in liver and heart. To investigate the role of Ex-FABP in vivo, we microinjected the affinity-purified anti Ex-FABP polyclonal antibody in chick embryo at early stage (23°-24°St). This treatment caused a drastic mortality of the embryo. The injection of Ex-FABP antibody directly conjugated with fluorescein showed that the specific target tissue is the heart. We analyzed the expression of apoptotic events in embryo injected with the antibody and we detected the presence of apoptotic cells in heart and liver tissues. We confirm that also in vivo the Ex-FABP acts as a constitutive survival protein and that its expression and activation are important to escape cell death.

Poster No. IV-7

Epitopic characterization of native bovine β-lactoglobulin.

Clement G*, Boquet D†, Frobert Y†, Bernard H*, Negroni L*, Chatel, J-M*, Adel-Patient K*, Creminon C†, Wal J-M* & Grassi J†, *Laboratoire d'immunoallergie alimentaire INRA-CEA and †Commissariat à l'énergie atomique CEA/DSV/DRM/SPI, Gif/Yvette Cedex, France

Many characterized animal aeroallergens belong to the lipocalin family as does the milk allergen β-lactoglobulin (BLG). If the linear T-cell epitopes of these allergens are known, nothing is known of their conformational B-cell epitopes. In this work, a panel of 52 monoclonal antibodies (mAbs) raised against BLG was studied. Firstly, an epitope map was drawn using a surface plasmon resonance (SPR) biosensor: the epitopes were organized in a circle of

11 overlapping antigenic regions and one non-overlapping. Secondly, fifty-five site-directed BLGA mutants were prepared and tested by ELISA and competitive immunoassay to localize these twelve antigenic regions on the protein molecule. Among them, twenty mutants showed a 10- to 7500-fold decrease in relative affinity for the mAbs of one or several neighbouring regions: their circular dichroism (CD) spectra were identical to the spectrum of wild-type (WT) BLGA. At least one mutant was found for each of the eleven overlapping antigenic regions which circled the molecule and for the non-overlapping one which was localized near the entrance of the calyx. Mabs had an high affinity for their epitopes (determined by ELISA and Biacore for one of them: $K_A = 2 \times 10^9\ M^{-1}$). A mutation in the β-sheet H - α-helix loop (E127A) caused the greater change in relative affinities of a mAb (a 7500 fold decrease). Strong effect mutations (4500 and 2000 decrease respectively) were also found in the α-helix (D130N and E134Q). Mutations were also found in β-sheets A and F (T18N and L93A) but they caused smaller decreases in affinity (200 and 300 respectively). These results show the first insight on the B-cell epitopic structure of a lipocalin.

Poster No. IV-8

Glycodelin S: Significance in Seminal Plasma

SundarRaj S, Mukhopadhyay D & Karande AA, Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Glycodelin, a homologue of β-lactoglobulin, is a lipocalin of the primate reproductive axis. Glycodelin A (GdA), the isoform in the female reproductive tract, has been well studied, and implicated in processes like fertilization, immunomodulation, and differentiation. However, the function of Glycodelin S (GdS), the major isoform in the male reproductive tract, is still a mystery. Unlike GdA, GdS does not interfere with sperm-zona binding. As the two isoforms have an identical aminoacid sequence but strikingly different glycosylation, it is inferred that glycosylation dictates the contraceptive function of glycodelin.

GdA is an indispensable molecule for the establishment, maintenance and progression of pregnancy. It can bring about a spatially and temporally regulated down-modulation of the maternal immune response. We have demonstrated that GdA mediates immunosuppression by inducing apoptosis in activated T-cells. To understand the role of GdS in the seminal plasma, where it is present in significant amount, we tested GdS for its ability to induce apoptosis in activated T-cells, and hence act as an immunosuppressive component. Our results show that GdS is not apoptogenic. Further, the difference in the sugars linked to the same protein backbone confers a slight yet functionally significant difference to the conformations of the two isoforms. The fucose rich GdS has a conformation that is relatively unstable and sensitive to trypsin cleavage, while the highly sialylated GdA is more stable, and resistant to trypsin cleavage.

We also see that GdS binds specifically to human spermatozoa. This, along with other preliminary data indicates that GdS may be involved in regulating sperm capacitation.

Poster No. IV-9

Effect of resveratrol on the expression of Neutrophil Gelatinase Associated Lipocalin (NGAL)

Roursgaard, M., Seidelin, M. & Vang O., Roskilde Universitetscenter, Roskilde, Denmark

Resveratrol is one of the most promising, naturally occurring anti-carcinogenic compounds. A cDNA array screening on the human colon cell line DLD-1 showed that among 1276 genes, Neutrophil Gelatinase Associated- Lipocalin (NGAL) was the most highly induced following exposure to resveratrol. The aim of this study is to identify the molecular mechanism for this induction of NGAL by resveratrol and to elucidate the molecular connection to cellular growth. This severe response was further confirmed and found to be time- and dose-dependent, at RNA and protein levels, RNA levels being induced 16 fold.

The expression of NGAL is likely to be regulated via the NF-κB pathway and several papers have identified that activation of NF-κB is inhibited by resveratroltreatment. To verify the possible role of NF-κB in the resveratrol mediated NGAL induction, DLD-1 cells have been transfected with plasmids encoding either normal IκB or mutated IκB. Mutated IκB can not be phosphorylated and thus inhibits the action of NF-κB. If resveratrol act via prevention of IκB inactivation, resveratrol will induce NGAL expression in cells transfected with wild type IκB but not in cells transfected with mutated IκB. The results will be presented.

The phorbol ester, TPA, increases cell growth and induces both NGAL and MMP-9 in a number of cell lines. Resveratrol block the TPA-induced growth of DLD-1 cells and the investigation of the antagonistic effects of TPA and resveratrol on the expression of NGAL and MMP-9 are in progress. In these experiments, we strive to identify a link between the resveratrol mediated NGAL-induction and cell growth inhibition.

Poster No. IV-10

Is Retinol-binding protein involved in transport to oocytes in freshwater and marine fish species?

Lubzens E, Lissauer L, Levavi-Sivan B, Avarre J-C & Sammar M, Israel Oceanographic and Limnological Research, Haifa, Israel

Fish yolk-laden eggs contain carotenoids, retinals and retinols that are utilized during embryonic development. While carotenoids are transported in the plasma of fish by lipoproteins (LDL, HDL and VHDL) or are associated with serum albumin and retinal is associated with vitellogenin, there is no information on the origin of retinol located in the oil

droplet fraction, obtained from homogenized eggs. Higher abundance of retinols and their esters was found in freshwater fish than in marine species, where they may reach 31-56% of the total retinoids. As retinol-binding protein (RBP) has been implicated as the main transporter of retinol to chicken oocytes, we examined whether RBP is involved in the transport of retinol to fish oocytes. Molecular characterizations were performed of RBP cDNAs from freshwater and marine fish species; trout, eel, the gilthead seabream and the white grouper. Comparison of the primary structure with those of Medaka, zebrafish, carp, *Xenopus,* crocodile, chicken and mammalian species revealed conserved characteristics in parallel to unique ones. All the six cysteines involved in disulfide bond formation are conserved. The differing features include; the signal peptide of fish differs from those of mammalian species, replacements of the amino acids comprising the Lipocalin domains and of those associated with binding to retinol. The functional significance of these differences remains unknown. Only 5 out of the 8 amino acid residues associated with the interaction of human RBP with TTR are conserved in fish RBPs, supporting studies on the monomeric appearance of fish holoRBP in the plasma. Since the glycolysation sites reported for carp RBP were not located in the other fish species, the pathway for retention of RBP in the plasma has yet to be determined. Expression and functional studies in fish may reveal the evolution and pleiotropic functions of RBP in vertebrates.

Poster No. IV-11

Engineering of Anticalins with Specificity towards an Oligohistidine-Sequence

Lazar Z, Weichel M & Skerra A, Institute of Biological Chemistry, Technical University Munich , Freising-Weihenstephan, Germany

Anticalins are artificial ligand-binding proteins derived from the lipocalin scaffold [1]. They represent an interesting alternative to antibodies with the advantage of being smaller in size and composed of a single polypeptide chain. In addition, they can be easily produced in a secretable form in *E. coli*, as functional fusion proteins with reporter enzymes [2], and even as so-called duocalins, i.e. functional fusion proteins of two different anticalins [3].

The goal of this study is the generation of an anticalin with specificity towards the hexahistidine tag, whose interaction is dependent on a transition metal ion (Ni^{2+}, Zn^{2+}, Cu^{2+}). Since the His_6-tag is widely used in molecular biology for the affinity purification of recombinant proteins such an anticalin would be suitable as a specific reagent for detection purposes. To this end 16 amino acid residues, distributed across the four loops of the ligand pocket of the bilin-binding protein (BBP), were subjected to random mutagenesis. From the resulting library an anticalin was selected using phage display and a filter-sandwich colony screening assay [2].

As measured in BIACORE experiments the variant exhibits a dissociation constant of ca. 2 μM for the His_6-tag as displayed by a recombinant protein (cystatin). The affinity depends on the concentration of the transition metal ion. Further improvement of the ligand affinity was achieved both via targeted randomisation and by means of error prone PCR. These anticalins show Ni-mediated affinity to several His_6-tagged recombinant proteins in the ELISA and on the Western blot and should thus be useful for practical application.

1. Skerra, A. (2001) *Rev. Mol. Biotechnol.* 74, 257-275.
2. Schlehuber, S., Beste, G. & Skerra, A. (2000) *J. Mol. Biol.* 297, 1105-1120.
3. Schlehuber, S. & Skerra, A. (2001) *Biol. Chem.* 382, 1335-1342.

Poster No. IV-14

C-terminal processing of a_1m in patients with hemolytic diseases

Nordberg $J^{1,2}$, Allhorn M^2, Åkerström B^2 & Olsson $ML^{1,3}$, [1]University Hospital Blood Centre, [2]Dept. of Cell and Molecular Biology and [3]Dept. of Transfusion Medicine, Lund University, Lund, Sweden.

Hypothesis: That α_1-microglobulin (a_1m) is processed *in vivo* into its heme-degradation form, t-a_1m, in patients with hemolytic and related diseases.

Background: a_1m is a plasma and tissue protein with heme-scavenging and heme-degradation properties. The protein is processed by ruptured erythrocytes and free hemoglobin *in vitro*, and a truncated form of a_1m, called t-a_1m, is formed, which lacks the C-terminal tetrapeptide LIPR. Both a_1m and t-a_1m binds heme and t-a_1m induces degradation of heme into a heterogeneous yellow-brown component strongly bound to the protein. a_1m is a low-molecular weight protein which passes the glomerular membranes of the kidneys, and normal human urine contains both full-length a_1m and t-a_1m.

Aim: To measure the ratio of full-length a_1m/t-a_1m in urine of patients with hemolytic disorders as compared to healthy individuals.

Methods: Affinity chromatography of urine from patients and normal blood donors, separation of a_1m/t-a_1m by SDS-PAGE and Western blotting using specific anti-LIPR antibodies.

Results: A limited study showed a relatively higher amount of t-a_1m in hemolytic patients (n=10) as compared to healthy individuals (n=10). This indicates that the cleavage of a_1m is a normally occurring biologic process and that t-a_1m may be increasingly produced during hemolysis *in vivo.*

Discussion: Heme and hemoglobin, which are exposed by cell damage, as for example in hemolysis, are sources of oxidative stress which may cause cell- and tissue damage. Our results suggest that, during hemolysis, free heme and hemoglobin induce an agent, t-a_1m, for its own degradation.