

Formålet med vores projekt var:

- At overføre og udnytte viden om en række fysiologiske og biokemiske processer i celler fra pattedyr til fiskeceller, samt at studere fysiologiske processer i fiskemusklens, specielt i forbindelse med iltmangel og reoxygenering. Dette har relevans for *post mortem* udviklingen af fisken og dermed for dens kvalitet som fødevarer. Formålet skulle opnås gennem etablering og studier af fiskecellekulturer fra væv af særlig betydning for fiskens kvalitet som fødevarer som f.eks. muskel, bindevæv og underhudsfedtvæv.
- At styrke den grundlagsskabende forskning i enzymet *trimethylamin-N-oxid aldolase*, som er ansvarlig for den meget uønskede dannelse af formaldehyd under opbevaring af fisk.
- At etablere et ph.d.-studieprogram i ”Fish muscle physiology and biochemistry” med henblik på at kunne tilbyde relevante kurser i et studieforløb for biokemikere og fysiologer med interesse for fisk samt for levnedsmiddelstuderende.

Vi havde følgende delprojekter (forløbet af disse vil blive beskrevet på engelsk):

### **1. Primary culture of myosatellite cells from rainbow trout and activation of whole-cell currents by chemical anoxia.**

Post Doc. Charlotte Hougaard, Ph.D. and Reader Else K. Hoffmann, Ph.D., August Krogh Institute, University of Copenhagen. (Charlotte Hougaard havde et 8 måneders post.doc.-stipendium under fishnet).

Background: To study the cellular signalling cascades activated during ischemia/anoxia in fish muscle cells, we aimed at establishing a permanent culture of myoblasts from rainbow trout as such a culture is not commercially available. The idea was that a culture rich in myoblasts could be established from a mixed culture of fibroblasts and myoblasts isolated from rainbow trout muscle by growing the culture under conditions where the growth of myoblasts is favoured.

Methods/Results: In the first attempts to establish such a culture, we used muscle tissue isolated from one-year old rainbow trout. The yield of myoblasts in these cultures were however very low (the myoblast/fibroblast ratio was approximately 1:25). It is known from other cell systems (e.g. rat) that the yield of myoblasts decreases as a function of age and thus to optimise the myoblast yield we tried to make primary cultures of myoblasts from newly hatched larvae and rainbow trout less than 4 months old. Several different methods were used in our attempts to establish a primary culture of myoblasts from larvae and young rainbow trout, including methods applied with success at the August Krogh Institute when establishing muscle-cell cultures from rats and the procedure used by Ma and Collodi (1999) to establish myosatellite cell cultures from lamprey. The best results were, however, obtained when using a slightly modified version of the method described by Matschak & Stickland (1995) to isolate the cells, and the method described by Koumans et al (1990) to enrich the percentage of myoblasts in the culture by adhesion to laminin-coated surfaces. In this culture the ratio between myoblasts and fibroblasts is, originally approximately 1:1. As fibroblasts and not myoblasts grow well in culture, the ratio will decrease rapidly and within weeks the culture appears to contain only fibroblasts. Although we have not been successful in establishing a pure myoblast culture it has been possible to perform electrophysiological single-cell experiments as the two types of cells are easily distinguished when looked at in a microscope.

Electrophysiological experiments were performed using the whole-cell patch-clamp technique. This method is extremely useful when studying ion transport via channels and has the great advantage that the cell interior can be controlled with regards to ion concentrations and thus the activated channels are easily distinguished. Anoxia was induced by exposing the cells to 10 mM Naazid in the presence of 10 mM glucose. This procedure inhibits the oxidative metabolism but ensures a certain energy level by glycolysis.

Exposing myoblasts to Na-azid (10 mM) induces a large outward whole-cell current and, in addition, the reversal potential of the current approximates the reversal potential for  $K^+$ , indicating that the current activated by Na-azid is primarily carried by  $K^+$  leaving the cell via  $K^+$  channels. At the present time it is unclear what types of  $K^+$  channels contribute to the Na-azid induced current in myoblasts isolated from rainbow trout, but preliminary investigations indicate that  $Ca^{2+}$ -activated and inwardly-rectifying  $K^+$  channels may contribute to the current (C. Hougaard, C. Ossum, E.K. Hoffmann, in prep). A few control experiments are still lacking.

## 2. Cell line development and signal transduction during chemically anoxic stress

Ph.D student Carlo G. Ossum and Reader Else K. Hoffmann, Ph.D.

**Aim of the studies.** The focus of this project has been the development of cell lines originating from edible tissue of the rainbow trout, *Oncorhynchus mykiss* W. and the studies of anoxia and reoxygenation of these cells.

**Results.** In order to study the effects of anoxia in piscine cell lines, anoxia was induced chemically by challenging the cells with sodium azide, which block the electron transfer to  $O_2$  in mitochondria. The cell line used is RTHDF. To this end, one fibroblast-like cell line from connective tissue of the hypodermis and surface of the muscle has been established, characterised and named RTHDF for Rainbow Trout Hypodermal Fibroblasts. In addition to an analysis of the basic properties of cell growth, serum requirements and detection of telomerase activity, the time-course of MAP kinase ( $p38^{MAPK}$ ) activation and expression profile of Hsp70 during anoxia and recovery were studied (C.G. Ossum, S.T. Christensen, E.K. Hoffmann, 2003; Ossum, C.G., Hoffmann, E.K., Vijayan, M.M., Holt, S.E. and Bols, N.C., 2004.). We found that sublethal anoxia rapidly activates  $p38^{MAPK}$  by phosphorylation and later induces an increase in the amount of the inducible heat shock protein Hsp70. We then studied the role of the MAPK family member extra cellular signal-regulated kinase, ERK1/2. ERK is typically activated by mitogens and play a crucial role for cell survival. We found that ERK is downregulated during anoxia and upregulated by subsequent reoxygenation. Upregulation of the ERKs by reoxygenation seems to be dependent on generation of reactive oxygen species. However, if the ERK-activity is completely abolished, the cells cannot be rescued by recovery (Ossum, C.G., and Hoffmann, E.K., 2005). In addition, experiments indicate involvement of PKC isoforms, although the role of PKC is still not clear. Another interesting observation is differences in isoform usage between the RTHDF cells and cells of mammalian origin, i.e. NIH 3T3 fibroblasts and Ehrlich ascites tumour cells; In the RTHDF cells, ERK1 is the preferred isoform, while in the mouse, ERK2 is predominantly activated.

Finally a new cell line – RTM C#2 – is under development. These cells are derived from rainbow trout muscle and appear to be fibroblastic.

**3A. Proteom changes in RTHD-cells after anoxia and reoxygenation analysed by 2D-gel electrophoresis.** Ph.D. Student Tune Wulff, Senior Scientist Flemming Jessen, Ph.D and Reader Else K. Hoffmann, Ph.D.

**Aim of the studies.** The aim of the studies is to investigate the proteom changes in various fish cell lines during anoxia.

**Results.** We have used two-dimensional gel electrophoresis to detect changes in protein expression. We were able to separate more than 1.500 protein spots with an apparent range of molecular masses from 10 to 120 kDa and *pI* values from 4 to 7.

Chemical anoxia obtained by addition of  $\text{NaN}_3$  is a widely used model for simulating anoxia, since azide inhibits the oxidative metabolism by blocking electron transfer between the cytochrome oxidase complex and oxygen (see e.g. Jørgensen et al. 1999). As a first part of the project we have investigated whether the differences in protein expression caused by chemical anoxia is the same as the changes caused by anoxia obtained by flushing the medium with nitrogen, and thereby evaluated chemical anoxia as a model for anoxia. In both cases the anoxic stimuli was followed by reoxygenation overnight. We found that the expression of more than 40 proteins changed after 30 minutes of chemical anoxia and subsequent reoxygenation overnight (CO). Two hours of anoxia obtained by flushing the medium with  $\text{N}_2$  followed by reoxygenation (AO) overnight, resulted in change of the abundance of more than 30 proteins. Surprisingly the results showed very little overlap between protein changes under the two conditions. Of all the protein changed under the two experimental conditions only five proteins have changed under both conditions. To further evaluate the differences between the two models, we used partial least squares regression to see which spots could be used to differentiate between the control situation, AO and CO. We were thereby able to build a model containing approximately 80 proteins which could be used to distinguish between the three situations. The study shows great differences in expression patterns depending of the nature of the stimuli, and one must therefore be careful when comparing results between the two models (**Wulff T., Jessen, F. and Hoffmann, E.K. in prep**). We are currently trying to identify the 40 proteins that changed after 30 minutes of chemical anoxia and subsequent reoxygenation overnight and the 30 proteins that had changed after two hours of anoxia obtained by flushing the medium with  $\text{N}_2$  followed by reoxygenation (AO). This is done by mass spectrometry in collaboration with prof Peter Roepstorff, University of Southern Denmark.

**3B. Proteome differences between families of reared rainbow trout**  
Senior Scientist Flemming Jessen.

White muscle protein expression in rainbow trout families was characterized by two-dimensional gel electrophoresis. Fish from 4 different families were compared. We found 30 proteins that differed significantly in expression between the 4 families revealing that family specific protein expression patterns exist. Even genetically closely related families (half-sib families) exhibit differences in their expressed protein patterns (**Leth, N.K., Jokumsen, A., Lund, I. and Jessen, F. 2005**).

#### **4. Biochemical and physiological properties of the enzyme responsible for formaldehyde formation in fish during storage.**

*Associate Professor, Vibeke Barkholt; Senior Scientist, Bo Jørgensen; Assistant Professor, Michael Nielsen*

Aim of the studies:

To perform basic research on the enzyme trimethylamine-N-oxide aldolase to avoid formaldehyde formation in stored fish.

Trimethylamine-N-oxide aldolase is an important enzyme from the point of view of applied research and should also be of great interest to basic research due to the vast lack of knowledge regarding

- **Biochemistry:** Size and amino acid composition. Reaction mechanism. Is it in fact a well-known metabolic enzyme with activity towards trimethylamine-N-oxide? Do different iso-enzymes exist?
- **Physiology:** What is its function in organs, muscle and gall juice? Why is it so unevenly distributed? Why is the difference among individuals so large?
- **Technology:** How can the activity be controlled (decreased) or at least estimated quickly using prior knowledge of e.g. fishing grounds, water temperature, season, age/size of the fish.

Results:

By the purification method developed during the project, a fairly pure enzyme preparation was obtained and the procedure used for isolating larger amounts of enzyme from saithe gall juice for future analysis. The enzyme activity in spleen and white muscle from a large number of species within the cod family was determined. The substantial intra-species, inter-fish variability and the big difference in activity between species were confirmed. The enzyme activity is not correlated to the muscle content of trimethylamine-N-oxide (**Nielsen, M.K. and Jørgensen, B.M., 2004,2005b**)

An idea that the *in vivo* biochemical function of the enzyme was connected to the choline metabolism has been explored, but the results were not fully conclusive.

Although the final enzyme preparation was suitable for kinetic measurements, it was not sufficiently pure for a thorough amino acid sequence analysis. The results obtained, however, to provide an important experience for future applications of various types of affinity chromatography to further purifying the enzyme (**Nielsen, M. K., Barkholt, V. and Jørgensen, B. M. (2005a)**)

Education:

Several Master Degree projects have been designed based on the project results with one Master Degree student graduating during the coming year. Kinetic work on the enzyme is also an integral part of a yearly Ph.D.-course organised by DTU and DIFRES.

#### **5. Osmotic stress and salt excretion in fish.**

C.G. Ossum and Else K. Hoffmann in collaboration with Prof. W.S. Marshall, Nova Scotia.

We used the Killifish (*Fundulus heteroclitus*) as our experimental model because it tolerates a wide range of salinities. In the sea it drinks sea water, absorb NaCl and water and excretes the extra NaCl over the gills. In fresh water it drinks little, excretes large quantities of dilute urine and absorbs ion via the gills. What happens then when the fish moves from salt – to fresh water and vice versa? When it moves from fresh water to sea water the plasma osmolarity rises from 300 mOsm to slightly over 400 mOsm and decreases again over more than 24 hours. From salt water to fresh

water plasma osmolarity decreases from 340 mOsm to 280 mOsm and rises again over 24 hours. We have studied the mechanisms involved in these adaptations both the salt transporting mechanisms in the gills and the cellular signalling mechanisms behind them (**Marshall, Ossum and Hoffmann, 2005**). Osmosensing in the gill cells of fish is an intriguing way to regulate salt excretion and it is very sensitive to lack of energy and thus to lack of oxygen as well as too many toxic compounds in extremely low concentrations. If the mechanism is harmed the fish will die of salt stress in salt water and of hyper hydration in fresh water.

## **6. Ph.D. course in fish muscle physiology and biochemistry** Danish Institute for Fisheries Research DTU, Lyngby, Denmark

Senior Scientist Flemming Jessen, Senior Scientist Bo M. Jørgensen and Assistant Professor Michael K. Nielsen.

Number of courses: 3

Number of participants: 42 (2002), 48 (2003) and 45 (2004).

Participants in practical courses: 12 (2002), 12 (2003) and 11 (2004).

The objective of these courses was to promote research in physiological processes in fish muscle that cause or influence post mortem changes, such as *rigor mortis*, changes in water holding ability, production of free fatty acids and break-down of proteins, in order to reveal its implications for fish processing and product quality .

The course comprised:

- a one-day seminar with lectures on selected topics in fish (muscle) physiology and biochemistry and
- 3½ days of exercises on post mortem processes.

International speakers at symposium:

2002

Prof. Ian A. Johnston, University of St. Andrews, Scotland

Dr. Richard Taylor, INRA de Theix, France

Dr. David J. McKenzie, University of Birmingham, England

Prof. Gert Flik, University of Nijmegen, The Netherlands

Prof. Geoffrey Goldspink, University Collage London, England

Dr. John Fleng Steffensen, University of Copenhagen, Denmark

2003

Dr. C. Louise Milligan, University of Western Ontario, Canada

Dr. Ian McCarthy, University of Wales, Bangor, United Kingdom

Dr. Richard Taylor, INRA, France

Dr Frank Bo Jensen, University of Southern Denmark, Denmark

2004

Prof. Andrew Cossins, University of Liverpool, UK

Prof. Bjørn Thrandur Björnsson, Göteborg University, Sweden

Prof. Kim Esbensen, Aalborg University Esbjerg, Denmark

Prof. Peter Belton, University of East Anglia, UK

Dr. Svante Winberg, Uppsala University, Sweden

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- Nielsen, M. K., Barkholt, V. and Jørgensen, B. M. (2005a)** Tentative title: “Preparation and characterisation of the formaldehyde-forming enzyme trimethylamine-N-oxid aldolase (EC 4.1.2.32) from various tissues of gadiforme fish.” (in prep)
- Nielsen, M. K. (2005b)** “Når fisken dør på en smagfuld måde”. Submitted June 2005 to *Fisk og Hav*, Danish Institute for Fisheries Research.
- Wulff, T., Jessen, F., and Hoffmann, E.K.** A comparative study in fibroblasts from rainbow trout of two different anoxia models by 2-dimensional gel electrophoresis. Under udarbejdelse.