Comparative iTRAQ™ based mass-spectrometry used to identify seasonal variation in the hepatoproteome of the dehydration and freeze tolerant wood frog *Rana sylvatica*

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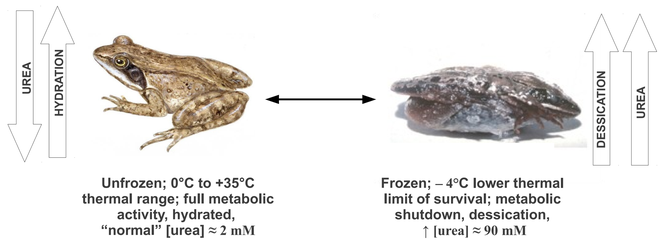


Introduction

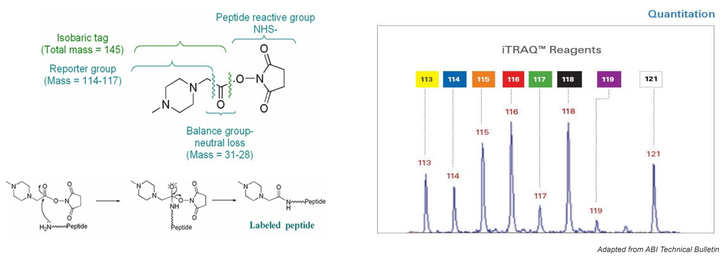
For many temperate animals, the advent of winter presents challenges including scarcity of food resources, reduced availability of environmental water, impairment of gas exchange, and declining temperatures. Physiological adaptation to these stresses often requires changes in gene expression that tune the quantities of various proteins to maintain cellular integrity and adapt metabolic processes. Such alterations become conspicuous in the proteome, and quantification of these changes can provide important insights into the adaptive processes functioning at multiple levels of biological organization.

Terrestrially-hibernating amphibians exhibit responses at molecular, cellular, and whole-organism levels that permit survival of prolonged aphagia, limited availability of environmental water, and potentially extreme hypothermia that may cause tissues to freeze. The roles of genes and their products in these survival mechanisms have received only cursory study, but may be expediently explored using mass-spectrometric-based proteomic profiling.

In this current project, we used liquid chromatography (LC) in combination with tandem mass spectrometry (MS/MS) quantitative isobaric (iTRAQ™) peptide mapping to investigate seasonal variation in the abundance of various proteins in liver of the wood frog, *Rana sylvatica*. Overwintering beneath duff in upland forests, these frogs survive many months without feeding and must endure dehydration and the freezing of up to 65-70% of their body water (1). Coping with these stresses requires induction of a host of adaptive responses incorporating molecular through to organismal levels of integration. Our aim in conducting this proteomics survey was to gain insights into the macromolecular responses at the tissue and cellular levels permitting winter survival in this species.

Methods

We isolated liver from two winter and two summer acclimatized frogs reared in our laboratory as previously reported (1). A portion of the medial lobe was ground under liquid N_2 and resuspended in PBS. Soluble protein (110 mg mL^{-1}) was then used for the subsequent iTRAQ™ labeling experiment (2). Isobaric tag for relative and absolute quantitation (iTRAQ™) 4-Plex labeling followed by 2D LC MuDPIT Tandem MS was performed at the Michigan Proteome Consortium on an ABI 4800 ToF/ToF Instrument.



Adapted from ABI Technical Bulletin

Figure 1: Principle behind iTRAQ labelling experiments for quantifying abundance of a given peptide in several experimental conditions. Note that after labelling the trypsin digested peptides with an iTRAQ reagent, multiple samples are combined and processed together.

Methods

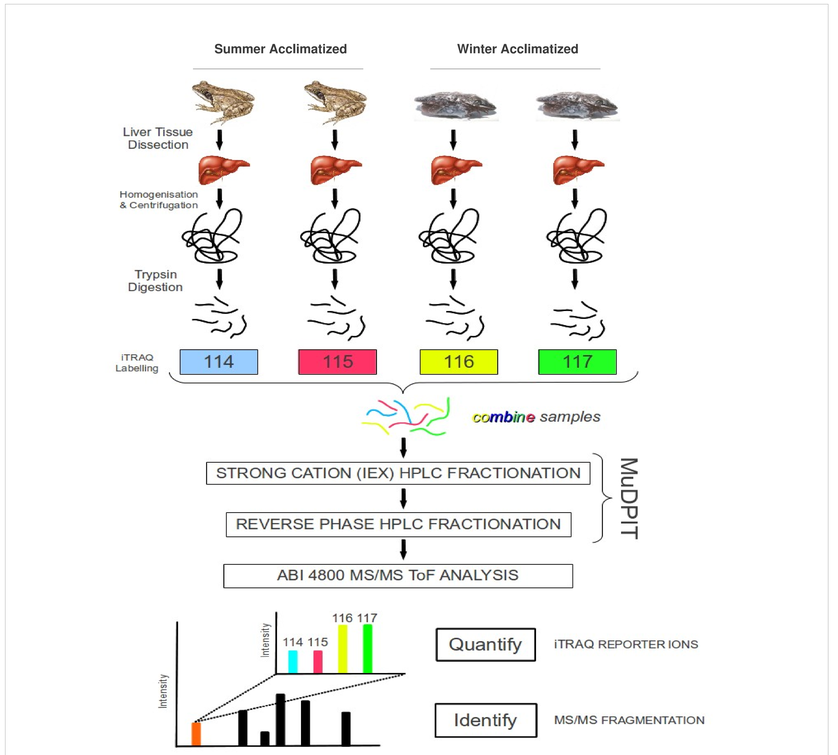


Figure 2: Work flow for the sampling of tissues for this project. Summer acclimatized and winter acclimatized (not frozen) frog's livers were sampled and homogenized under liquid N2. Homogenates were suspended in PBS and centrifuged, with soluble homogenates used for iTRAQ labeling and downstream MS/MS analysis.

iTRAQ = "*isobaric tags for relative and absolute quantitation*"

iTRAQ is a method and approach that permits an investigator to determine very accurately the amount that a given protein or peptide changes between experimental conditions (Fig. 2). Because the labeling step is done on separate experimental treatments and then combined, the mass-spectrometry analysis is very reproducible within a given analytical run. In other words, issues with run to run variability are eliminated.

Within each experimental condition, a reporter group (113, 114, 115, 116, 117, 118, etc) as part of an isobaric tag is chemically attached to the mixture of proteins (Fig. 1). Each isobaric tag has a unique reporter group plus a variable "balance" group (depending on the mass of the tag, *i.e.* [113 + 32] such that the overall addition to the protein or peptide is isobaric (the same mass, *i.e.* 145) in all experimental conditions. During disintegration in a collision cell (the second MS of the "MS/MS") in a Tandem ToF instrument, the balance group is cleaved and the unique reporter group remains attached to the protein/peptide from the given experimental treatment(s) (Fig. 1 & 2).

Results

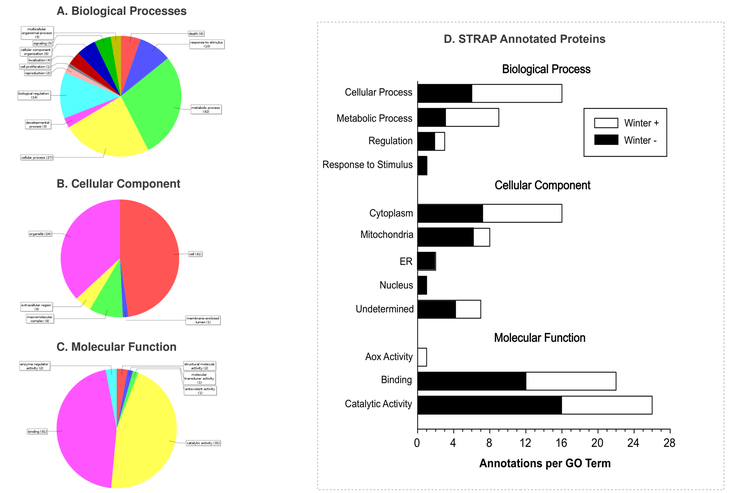


Figure 3: Identified proteins from livers of summer and winter-acclimatized wood frogs (*Rana sylvatica*) as annotated by *Blast2GO* (2) and *STRAP* (3), both of which use the NCBI GEO DB. Blast2GO identified and classified the proteins into three major categories of (A) Biological Processes, (B) Cellular Component and (C) Molecular Function. Annotation by the more conservative STRAP (D) of 33 differentially-expressed proteins was used to construct a stacked histogram.

Table 1: Proteins identified from extracts of liver from *Rana sylvatica* that were LESS abundant in winter than in summer.

GI	Protein	ARs	Species	Function
129730	protein disulfide isomerase precursor	2.34	*Oryctolagus cuniculus*	protein folding
9755362	acetaldehyde dehydrogenase	2.28	*Mus musculus*	alcohol metabolism
85719973	peptidylprolyl isomerase B	2.22	*Ictalurus punctatus*	protein folding
57087309	mRNA transport regulator 3	1.74	*Canis familiaris*	protein synthesis
47498070	aspartate aminotransferase 1	1.68	*Xenopus tropicalis*	amino acid metabolism
146415164	RNA recognition motif (hypothetical)	1.65	*Pichia guilliermondii*	protein synthesis
5921957	carbamoyl-phosphate synthase I	1.53	*Rana catesbeiana*	urea metabolism
148226795	binding immunoglobulin protein (BiP)	1.51	*Xenopus laevis*	protein folding/stress
121594245	protein DUF891	1.51	*Acidovorax* sp.	unknown
47210694	glutamate dehydrogenase 1	1.49	*Tetraodon nigroviridis*	nitrogen metabolism
45383354	histidine ammonia-lyase	1.38	*Gallus gallus*	amino acid metabolism
124266729	acetyl-CoA C-acetyltransferase 1	1.34	*Methylibium petroleiphilum*	protein/lipid metabolism
58332740	aconitase 2	1.33	*Xenopus tropicalis*	TCA cycle
148230238	homogentisate 1,2-dioxygenase	1.32	*Xenopus laevis*	amino acid metabolism
126632707	long-chain acyl-CoA dehydrogenase	1.26	*Danio rerio*	fatty acid metabolism
50417404	alanine-glyoxylate aminotransferase	1.24	*Xenopus laevis*	amino acid metabolism
56377788	elongation factor 1-α	1.22	*Pelodiscus sinensis*	protein synthesis
89886140	phosphoenolpyruvate carboxykinase1	1.21	*Xenopus tropicalis*	gluconeogenesis

Table 2: Proteins identified from extracts of liver from *Rana sylvatica* that were MORE abundant in winter than in summer.

GI	Protein	ARw	Species	Function
147901600	glycogen phosphorylase	2.04	*Xenopus laevis*	glycogen catabolism
145545139	protein kinase C	1.72	*Paramecium tetraurelia*	signal transduction
148227386	pyruvate carboxylase, gene 1	1.67	*Xenopus laevis*	gluconeogenesis
134254218	pyruvate carboxylase, gene 2	1.51	*Xenopus tropicalis*	gluconeogenesis
118083730	1,4-α-glucan branching enzyme	1.42	*Gallus gallus*	glycogen synthesis
63146078	heat shock protein 70 (hsp70)	1.34	*Oxyuranus scutellatus*	stress response
2196882	heat shock cognate protein 70 (hsc70)	1.32	*Pleurodeles waltl*	protein folding
148225037	pyruvate kinase type M2	1.32	*Xenopus laevis*	glycolysis
147902026	peroxiredoxin 6	1.30	*Xenopus laevis*	antioxidation
91084329	protein phosphatase-1 α	1.29	*Tribolium castaneum*	glycogen metabolism
148234835	β-ureidopropionase	1.27	*Xenopus laevis*	AA metabolism
54020777	hydroxyacyl-CoA dehydrogenase	1.24	*Xenopus tropicalis*	FA metabolism
148224534	Arp2/3, subunit 2	1.24	*Xenopus laevis*	actin polymerization
148223127	glyceraldehyde-3-phosphate dehydrogenase	1.21	*Xenopus laevis*	glycolysis; non-metabolic processes

AR = Abundance Ratio
ARs = protein abundance in summer frogs relative to that in winter frogs (Table 1).
ARw = protein abundance in winter frogs relative to that in summer frogs (Table 2).

Summary

Most proteins (19 or 58%) were reduced in abundance in winter relative to summer. This group included mitochondrial enzymes as well as proteins involved in cell growth and proliferation (Table 1). The smaller set (14 or 42%) of up-regulated proteins contained relatively few metabolic enzymes and was dominated by proteins important to preserving cellular integrity and function (Table 2).

Several proteins with anti-oxidation properties have been previously observed to increase during chilling or dormancy (4), and we observed in winter-acclimatized frogs an increased levels of peroxiredoxin, a member of the ubiquitous family of sulfhydryl-linked antioxidants that can become a multimeric chaperone during oxidative stress.

Enzymes mediating the interconversion and catabolism of amino acids, nitrogenous waste formation were down-regulated in our winter-acclimatized frogs as well (Table 1). Glycogen phosphorylase, on the other hand, was elevated presumably to quickly mobilize glucose in response to tissue freezing.

Interestingly, we found that there were reduced levels of cataplerotic enzymes, including PEPCK, but increased levels of pyruvate carboxylase, suggesting an accumulation of oxaloacetate (OAA). OAA is a known inhibitor of CII, thus maybe an adaptation to minimize oxidative damage during the freeze/thaw process.

References

(1) Costanzo, J.P.; Lee, R.E. Cryoprotection by urea in a terrestrially-hibernating frog. *Journal of Experimental Biology* 2005, *208*, 4079-4089.
(2) Götz, S., García-Gómez, J.M., Terol, J., Williams, T.D., Nagaraj, S.H., Nueda, M.J., Robles, M., Talón, M., Dopazo, J. and Conesa, A., 2008. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 36, 3420-3435.
(3) Bhatia, V.N.; Perlman, D.H.; Costello, C.E.; McComb, M.E. Software tool for researching annotations of proteins: open-source protein annotation software with data visualization. *Analytical Chemistry* 2009, *81*, 9819-9823.)
(4) Carrasco, M.A.; Tan, J.C.; Duman, J.G. A cross-species compendium of proteins/gene products related to cold stress identified by bioinformatic approaches. *Journal of Insect Physiology* **2011**, *57*, 1127-1135.